THE BIOSYNTHESIS OF EXTRACELLULAR POLYSACCHARIDES IN *KLEBSIELLA AEROGENES*

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SUMMARY

The cultural characteristics of the capsulate strains K. aerogenes A1 and A4 were discussed, and their mutation to strains which had lost the ability to synthesize exopolysaccharide. The presence of a capsule was shown to protect the micro-organism against dessication, against attack by organic cations and enzymes degrading the cell wall, and against phage. A mutant of A1 was found, A1S1, which produced exopolysaccharide in the form of slime. The exopolysaccharides were purified and analysed. Incubation of the cells with C^{14}-glucose-6-phosphate led to labelling of all the components of the polysaccharides. Total polysaccharide production was estimated during growth, and a comparison was made using washed cell suspensions between the rate of exopolysaccharide synthesis by A1S1 cells grown for various times in different media.

The levels of nine enzymes involved in synthesis of the sugar nucleotide precursors of the exopolysaccharides were assayed in strains A1, A1S1 and A4. Their specific activities were also established in cultures of various ages, or grown at different incubation temperatures, or grown in different media which resulted in a variation in exopolysaccharide synthesis. There was generally little change in the level of any enzyme assayed.

Non-mucoid (0) mutants of strains A1, A1S1 and A4 occurred spontaneously at low frequency and this rate was increased by use of mutagens such as 2-aminopurine and acriflavine. Attempts to obtain reversion of these mutants were unsuccessful. There was, however, some evidence that the ability to synthesize the exopolysaccharide was regained in a few A1(0) strains by transduction. Using A1(0), A1S1(0) and A4(0) mutants, the levels of several enzymes synthesizing the nucleotide sugar precursors of the exopolysaccharides were assayed and compared

to the parent strains. No enzyme was deficient and generally their specific activities were the same as the parent.

A variant of A1 was found which was very unstable, giving rise to mucoid and non-mucoid cells with equal frequency, and a mutant of A4 produced far more exopolysaccharide when grown on galactose as carbon source than on glucose. It appeared to be deficient in the enzyme UDPG pyrophosphorylase.

A new class of mutants was discovered, the CR mutants, in which the presence of the exopolysaccharide was temperature dependent. It was only synthesized at incubation temperatures above 30°. There was a second effect on lipopolysaccharide synthesis which led to altered phage sensitivity patterns, to the characteristic crenated appearance of colonies, and to autoagglutinability in liquid culture at low incubation temperatures. Double mutants, which retained the CR appearance at all incubation temperatures but which had lost the ability to synthesize the exopolysaccharide, were also isolated. The properties of these mutants and the nature of the mutation were discussed.

Finally a system was developed in which cell-free synthesis of the A4 exopolysaccharide from the labelled nucleotide sugar precursors occurred. Good incorporation of glucose from UDPG into polysaccharide material was obtained. After pre-incubation of the system with UDPG, galactose from UDPGal was incorporated to one-half this extent, and glucuronic acid from UDPGlUA to one-tenth this extent. Chloramphenicol stopped the incorporation of glucose into polysaccharide material. The possibility of lipid-linked intermediates being involved was considered, and the effect of ADPG on the system found.

C O N T E N T S

INTRODUCTION

SECTION A

GENERAL INTRODUCTION

Polysaccharides are large macromolecular structures which consist of subunits of simple sugars or their derivatives joined by glycosidic linkages. All bacteria synthesize them at some stage during growth and, just as forms of microbial life tend to be diverse, so the polysaccharides are very varied. They may be produced intracellularily and extracellularily in addition to being part of the structure of the cell.

The cytoplasm and nucleus of the bacterial cell are separated from the environment by a membrane composed of protein and lipid. This acts as the semipermeable and selective barrier of the cell. In most cases it is surrounded by a thicker structure - the cell wall. The wall is thought to give the organism its shape and rigidity and to protect the membrane below it. Its essential function is to prevent lysis due to high internal osmotic pressure if the organism is placed in a hypotonic environment. Macromolecules which are cross linked and of high tensile strength are required so that the membrane cannot swell beyond a certain limit. Polysaccharides fulfill these physical requirements and have been found as part of all cell walls. The particular polysaccharide used is characteristic of the organism. Cellulose, a polymer of glucose, is found in cell walls of plants and green algae; chitin, a polymer of N-acetylglucosamine in fungi. The polysaccharide of the cell walls of bacteria is distinctive and probably unique to this group. It is a mucocomplex of amino sugars and amino acids. It is generally complexed

with other polymers in the cell wall.

Many bacteria are characterized by their ability to produce extracellular polymers of high molecular weight. They have limited solubility in water and accumulate in the medium around the cells. They may either form a definite layer covering the cell wall, in which case the organism is said to be capsulate, or be produced as slime which forms a diffuse mass in the medium. The extracellular material may be polysaccharide in nature, consisting of simple sugars, their acids or amino sugars, or polypeptide, like the capsule of Bacillus anthracis (Ivanovics and Bruckner, 1937), or a combination of both, like the capsule of B.megaterium (Guex-Holzer and Tomcsik, 1956). More than one type of polymer may be elaborated by the cell. The presence of such a polymer may be recognized readily by the mucoid, slimy appearance of colonies on solid media and by the increased viscosity and gelatinous consistency of liquid media. The polysaccharide nature of microbial slime material has been recognized for over a hundred years (Pasteur, 1861).

In a variety of bacteria, polysaccharide may be found also within the membrane forming a cellular storage material, a reserve of carbon and energy.

Until recently methods of extraction, purification and analysis of these polysaccharides were poor. However techniques have been developed which resulted in the discovery of new polysaccharides, their component sugars and, in a few cases, their structure. In addition the development of the electron microscope has allowed study of the fine structure of the cellular organization.

EXTRACTION AND PURIFICATION OF POLYSACCHARIDES.

Generally extracellular polysaccharides are not strongly attached to the

cell surface and may be separated from the cells by centrifugation leaving the polysaccharide in the supernatant. In the case of capsular material, the cells may be shaken in water or buffers, treated with dilute alkali, or boiled prior to centrifugation. The polysaccharide may be precipitated from the supernatant by acetone or alcohol.

Isolated cell walls may be prepared by mechanical disintegration and differential centrifugation (Salton and Horne, 1951) and purified by treatment with various enzymes to free them from cell wall proteins and cytoplasmic materials (Cummins, 1956; Cummins and Harris, 1956).

The polysaccharides of the cell walls are more firmly attached than the extracellular polymers and require some kind of chemical extraction procedure to separate them from cell walls or whole cells. Trichloracetic acid is often used (Boivin, Mesrobeanu and Mesrobeanu, 1933). This extracts the O-antigenic polysaccharides together with lipid and protein from Gram-negative bacteria, and the polyol phosphate when present from Gram-positive bacteria. Precipitation is then carried out with alcohol or ammonium sulphate. A milder method of extraction involves the use of diethylene glycol but its application is limited to a few bacterial species (Morgan, 1937). The lipopolysaccharide complex may be extracted from Gram-negative cells using 45% phenol at 60° (Westphal, Lüderitz and Bister, 1952). Lipopolysaccharide and RNA are contained in the aqueous phase leaving protein and cell debris in the phenol phase. Lipopolysaccharide and RNA can then be separated by ultracentrifugation. Mild acid hydrolysis is used to split polysaccharide from lipopolysaccharide (Morgan and Partridge, 1940).

Separation of protein and polysaccharide may be carried out by the method of Sevag (1934). Mixtures of polysaccharides may be resolved by use of long chain quaternary ammonium compounds such as "cetavlon" which complexes with acidic polysaccharides and precipitates at varying salt concentrations; neutral polysaccharides will not complex (Scott, 1960).

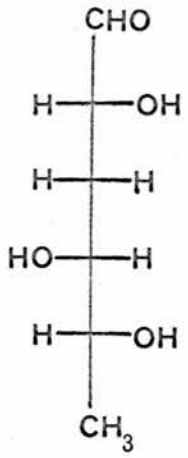
Tests for homogenity of the polysaccharide preparation should be made before chemical analysis. Methods used include electrophoresis (Northcote,1954), ultracentrifugation (Davies, Morgan and Record, 1955) and serology (Kabat and Mayer, 1948).

MONOSACCHARIDE UNITS OF POLYSACCHARIDES.

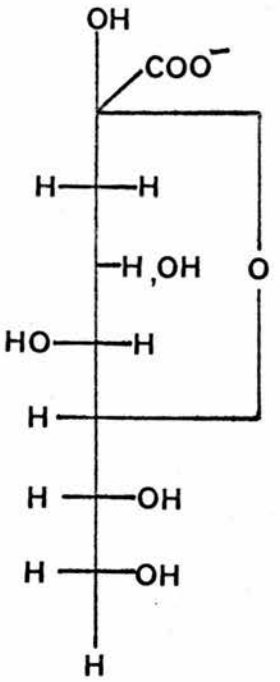
The range of sugars known to be present in polysaccharides has greatly increased in recent years due mainly to improvement in chromatographic techniques. Now more than twenty monosaccharides are known.

In a review in 1946, Stacey noted the presence in various polysaccharides of the hexoses, D-glucose, D-galactose, D-fructose and D-mannose together with the pentose D-arabinose, 2-amino-2-deoxy-galactose and glucose, glucuronic acid, galacturonic acid, L-fucose and L-rhamnose. A few years later a new series of sugars was discovered in hydrolysates of O-antigenic material of Salmonella species which were characterized as 3, 6-dideoxyaldohexoses (Westphal, 1952; Pon and Staub, 1952) and four have been described - abequose, paratose, tyvelose and colitose. Aldoheptoses are now known to occur in Gram-negative cell walls. D-glycero-L-manno-heptose was first isolated from the lipopolysaccharide of Shigella sonnei (Jesaitis and Goebel, 1952). Since then L-glycero-D-mannoheptose has been found in S.flexneri (Slein and Schnell, 1953) and Escherichia coli (Weidel, 1955). Aldoheptoses

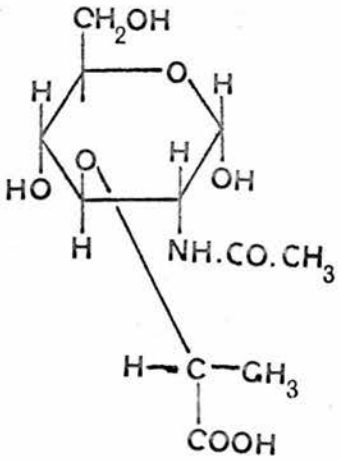
FIG 1



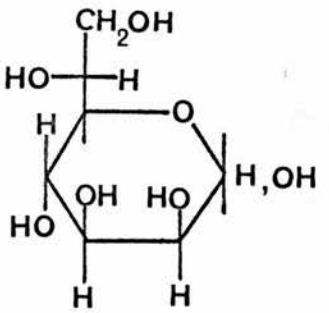
3,6-dideoxygalactose (abequose)



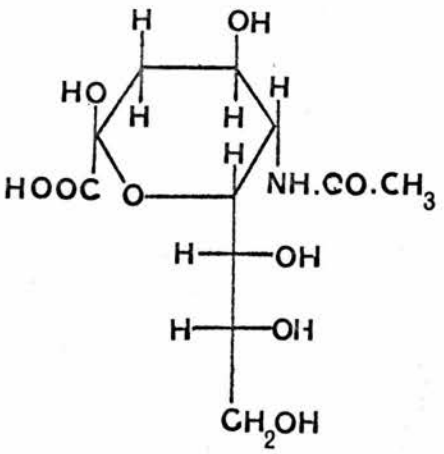
2-keto-3-deoxyoctonate



N-acetylmuramic acid



L-glycero-D-mannoheptose



N-acetylneuraminic acid

have also been found in Chromobacteria and Pasteurella species in addition to Enterobacteriaceae and twelve of the sixteen possible aldoheptose configurations have been recorded. Muramic acid has been found as part of the cell wall of all bacteria (Weidel and Primosigh, 1957), and neuraminic acid (review, Zilliken and Whitehouse, 1958), both of which are 9 carbon compounds. 2-keto-3-deoxyoctonic acid (KDO) has been discovered as part of the lipopolysaccharide of the cell wall (Heath and Ghalambor, 1963) being first found as the phosphate derivative in extracts of Pseudomonas aeruginosa (Levin and Racker, 1959). Finally the sugar alcohols, ribitol and glycerol have been characterized from the polyol phosphate of the Gram-positive cell wall (Baddiley, 1962).

The structures of some of these monosaccharides are illustrated in Fig.1 and the subject has been reviewed by Davies (1960).

Extracellular polysaccharides are produced by a variety of Gram-positive and Gram-negative organisms and range from homopolymers, such as the cellulose produced by Acetobacter xylinum, to heteropolymers, such as the polysaccharide of E.coli K27 containing glucose, galactose, glucuronic acid, fucose and O-acetyl groups (Jann et al, 1968). Others are complexed with additional material like polypeptide in B.megaterium (Tomcsik, 1951) or fatty acid and protein in Serratia marcescens (Adams and Young, 1965).

Cell walls represent a major fraction of the dry weight of the cell and analyses of isolated cell wall preparations showed both similarities and differences between Gram-negative and Gram-positive bacteria. The former contain a variety of amino-acids like those found in many proteins, while the latter contain only three or four amino-acids and of these, none is aromatic or sulphur - containing. Another difference is apparent in lipid

content. Walls of Gram-negative organisms contain up to 20% lipid, while the Gram-positive contain only very small amounts, around 1% (Salton, 1956). One polysaccharide was found to be shared by both types of cells, although it accounted for a much smaller proportion of Gram-negative cell walls. This has been called variously mucopeptide (Mandelstam and Rogers, 1959), peptido-polysaccharide (Sharon, 1963) or murein (Weidel and Pelzer, 1964; Martin, 1966). It consists of two amino sugars, muramic acid isolated in 1956 (Strange and Dark) and N-acetylglucosamine together with three, four or five amino acids. It is responsible for the mechanical properties of the cell wall. The other polymers may supplement this, provide surface antigens and protect it physically and from attack by phage. The cell walls of Gram-negative organisms contain in addition to murein, phospholipid, polysaccharide and protein. These are thought to form a lipophilic complex enveloping the murein (Lüderitz, Jann and Wheat, 1968). The lipopolysaccharide part has received much attention as it contains the O-antigenic specificity of the cell and its endotoxic properties. The polysaccharide is complex consisting of up to ten monosaccharide components which form three regions (see section D). In Gram-positive organisms, the other main polymer apart from murein is a polyol phosphate called teichoic acid (Armstrong, Baddiley, Buchanan and Carss, 1958). It contains either ribitol or glycerol and has not been reported in Gram-negative cell walls except in one case where a ribitol teichoic acid was present in a strain of E.coli (Lilly, 1962). It may act as the equivalent of the lipopolysaccharide, being an important antigenic determinant. A variety of neutral sugars are found in addition in Gram-positive cells and these may characterize a particular genus (Cummins and Harris, 1956), for example rhamnose and glucosamine in Streptococci (McCarty, 1952).

These sugars may be linked covalently to murein. The cell walls of most Gram-positive bacteria contain no protein material although it has been reported in a few instances in surface layers, (Janczura, Perkins and Rogers, 1961; Yoshida et al, 1957). Differences in fine detail between cell walls of Gram-negative and Gram-positive bacteria may be seen in electron microscope preparations. The cell walls of the former consist of several layers, the inner thought to be murein and the outer lipopolysaccharide-protein-lipid. It is difficult to distinguish membrane from wall. In the latter, only one layer is seen and the membrane is quite distinct from it. The space between is often crossed by narrow bridges which may be extensions of the membrane (Glauert, Brieger and Allen, 1961).

The intracellular polysaccharide is composed of glucose. It may be synthesized by both Gram-positive and Gram-negative bacteria, in quantities up to 20% of the dry weight of the cell depending on the growth conditions (Sigal, Cattanéo, and Segel, 1964). It is present as granules of small size.

SYNTHESIS OF MONOSACCHARIDE PRECURSORS.

The monosaccharide components of the polysaccharides are seldom found as free sugars. They may be complexed as glycosides, oligosaccharides and polysaccharides and, if supplied to organisms, will enter the general metabolism before incorporation into polysaccharides. If degradation of the polysaccharides occurs, they may be formed transiently.

During early work the precursors of the polysaccharides were not known. It was generally assumed that they would be built up from the monosaccharides supplied to the organism by a reversal of catabolic reactions. Thus glycogen was synthesized from glucose (Croft Hill, 1898) and levan from sucrose

(Beijerinck, 1910). However, amounts of monosaccharides required for these polymerizations were too large to be formed *in vivo*.

It was not until the early 1950s, work done by Leloir's group showed how monosaccharides were transformed within the cell and used as precursors for polysaccharide synthesis. This followed from the research of Cori, Schmidt and Cori (1939) who first envisaged the concept of transglycosidation from a phosphate ester of the sugar. The enzyme phosphorylase in liver had been found to convert glycogen to glucose-1-phosphate provided inorganic phosphate was present. It would also catalyse the reverse reaction forming chains of glucose units. The synthesis of starch in plants was found to occur similarily (Hanes, 1940). A few years later dextran (Hehre and Sugg, 1942) and levan (Hestrin, Avineri-Shapiro and Aschner, 1943) were synthesized enzymatically *in vitro* using extracts prepared from culture filtrates or ground cells. It was known that sucrose, the precursor, could be phosphorylased in the presence of inorganic phosphate to glucose-1-phosphate. But this was not shown to be an intermediate in formation of either polymer (Hehre, 1943). Then a group of enzymes, the pyrophosphorylases, was found which could catalyse the formation of disaccharides from monosaccharides, such as sucrose from D-glucose-1-phosphate and D-fructose (Doudoroff, Barker and Hansh, 1947). It was concluded that transglycosidation from sugar phosphates to appropriate acceptors may be a general mechanism of polysaccharide synthesis, although in other systems phosphorylases were inactive and no synthesis could be obtained

Leloir and his colleagues discovered the presence of a new class of sugar phosphate compounds which explained the previous results. They had been studying the utilization of galactose by the yeast *Saccharomyces fragilis*

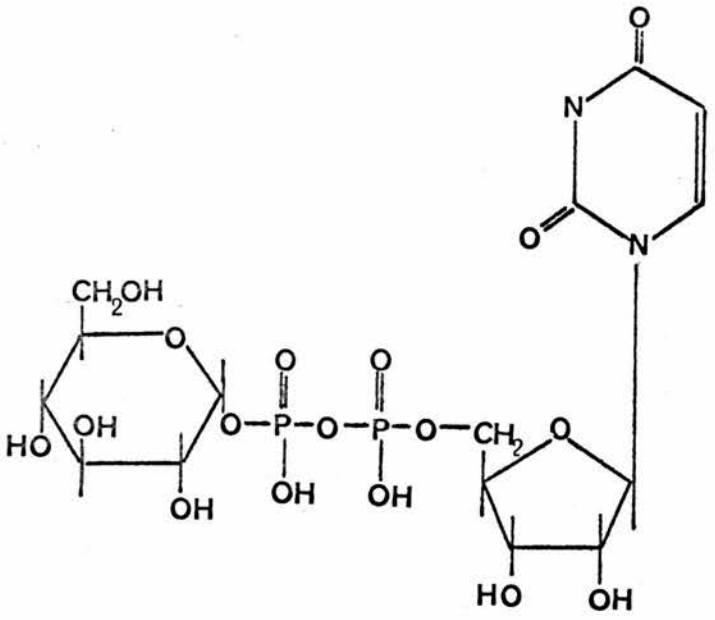


FIG 2

Uridine 5′-diphosphate glucose.

and found that cell free extracts would catalyse the conversion of galactose-1-phosphate to glucose-1-phosphate. The co-factor involved was isolated and discovered to be one of a new class of compounds - the sugar nucleotides. It was called uridine diphosphate glucose (Cardini, Paladini, Caputto, Leloir and Trucco, 1949; Cardini, Paladini, Caputto and Leloir, 1950). It was shown to participate in formation of uridine diphosphate galactose (Leloir, 1951). Its structure is shown in Fig.2.

Independently a new group of sugar nucleotides was isolated from penicillin treated cells of Staphylococcus aureus by Park and Johnston (1949). They were later identified as UDPNacetylmuramic acid and UDPNacetylmuramic acid peptides (Park, 1952). After the structure of the cell wall had been worked out in some detail, they were suggested to be precursors in its biosynthesis (Park and Strominger, 1957).

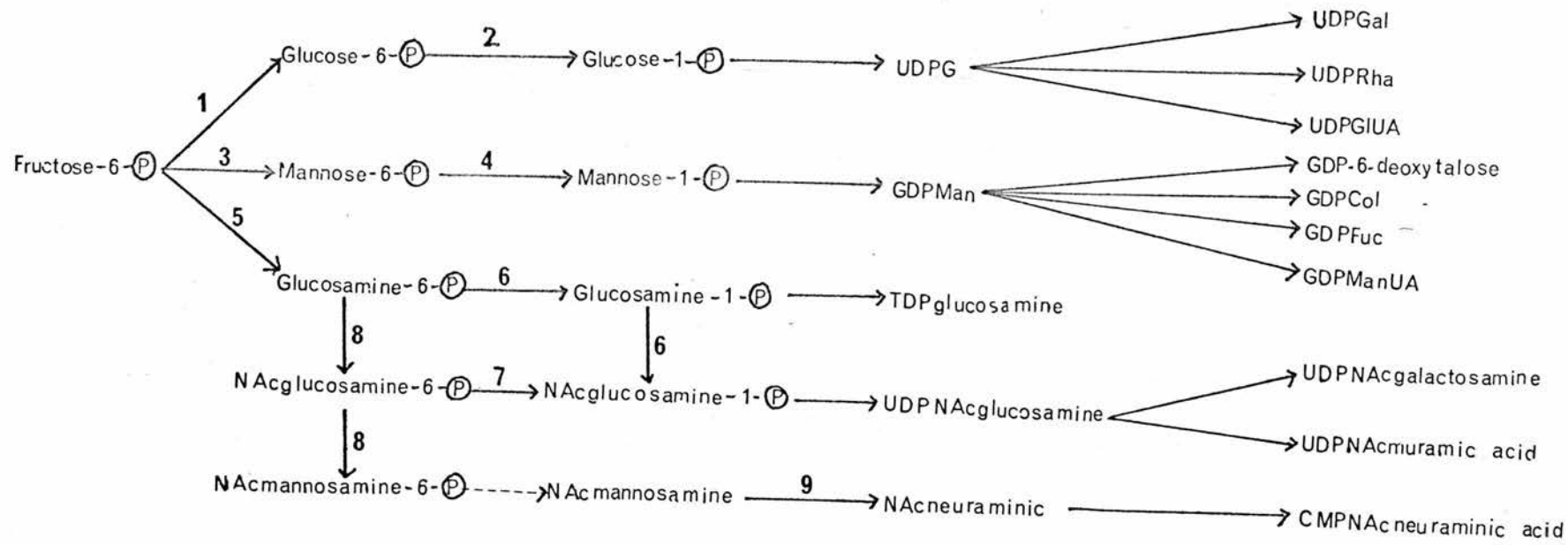
In addition to being involved in sugar transformations the nucleotide sugars were demonstrated to be glycosyl donors. In synthesis of β-O-amino phenol glucuronide using liver homogenate as crude enzyme, glucuronic acid was transferred from UDP glucuronate to the acceptor O-aminophenol (Dutton and Storey, 1953). Also UDP glucose was shown to be a glycosyl donor in synthesis of trehalose phosphate (Leloir and Cabib, 1953).

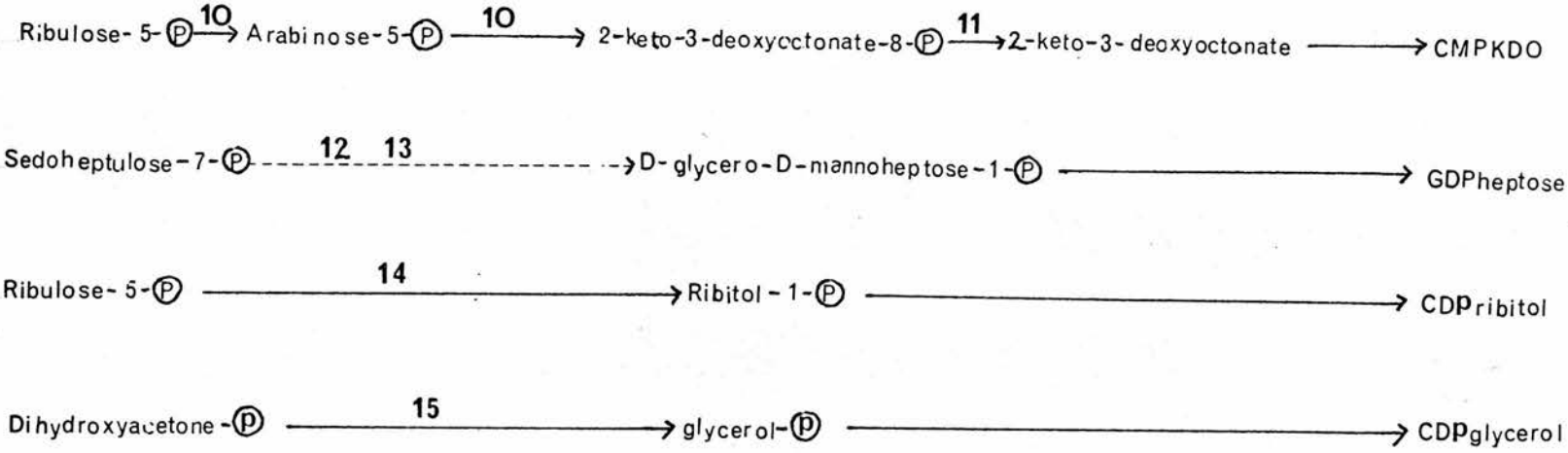
$$Glc - 6 - \textcircled{P} + UDPG \longrightarrow Glc - Glc - 6 - \textcircled{P} + UDP$$

Later the sugar nucleotides were shown to be involved in synthesis of hyaluronic acid, a polymer of N-acetylglucosamine and glucuronic acid, by Group A streptococci (Glaser and Brown, 1955).

So it is now thought that polysaccharide synthesis involves glycosyl transfer from sugar nucleotides to appropriate acceptors. Also it is assumed

FIG 3 Synthesis of Monosaccharides and some of their Nucleotide Derivatives





(Figures indicate references)

that if a sugar is found in a polysaccharide, it will also be found as the nucleotide derivative. The discovery of the nucleotide has even preceded its isolation in a polysaccharide as is the case with CDPglycerol and CDPribitol, precursors of the teichoic acid polymers (Baddiley, Buchanan, Carss and Mathias, 1956; Baddiley, 1962).

The pyrophosphorylases catalyse synthesis of nucleotide sugars by transfer of a nucleotidyl group from a nucleotide triphosphate to a sugar phosphate with formation of inorganic pyrophosphate.

For example $Glc-1-\textcircled{P} + UTP \longrightarrow UDPG + PP$

Unless a specific kinase and pyrophosphorylase are available, sugars supplied to the organism will enter the general metabolism and will not be converted directly to the nucleotide sugar. For example, animals are unable to use glucuronic acid directly for synthesis of glucuronides (Douglas and King, 1953), but in plants there is a specific kinase and pyrophosphorylase for this purpose and glucuronic acid may be used directly (Hassid, Neufeld and Feingold, 1959). The monosaccharides may be found therefore only as nucleotide derivatives. They can undergo many transformations including epimerization, oxidation, reduction, decarboxylation. These are reviewed by Ginsburg (1964). Synthesis of monosaccharides and some of their nucleotide derivatives are illustrated in Fig.3.

The nucleotides UDPG, GDPM and UDPNacetylglucosamine are found in all organisms. This indicates their importance as intermediates and precursors of other monosaccharides. For example UDPG may be transformed to UDPGal, UDPGlUA, UDPGalUA, UDPIUA, UDPXyl, UDPAbe. Epimerization may occur within UDPG or GDPM at C3, C4, or C5 but only in one reported case at C2 (Baddiley, Blumson, Girolamo and Girolamo, 1962). It is thought therefore that D-sugars

may be derived from UDPG and L- from GDPM.

Of the pyrophosphorylases, UDPG pyrophosphorylase is found universally. Other pyrophosphorylases which incorporate Glc-1-Ⓟ into additional nucleotides occur including guanosine, adenosine, cytidine and deoxythymidine but these are not distributed widely. The idea has been put forward (Ginsburg, 1964) that the occurrence of different nucleotide bases for the same sugar may afford a separation of their biosynthetic pathways and independent control. Thus in the O-antigenic polysaccharide of Salmonella typhimurium which contains rhamnose, galactose, abequose and mannose, mannose is derived from the guanosine nucleotide, abequose from cytidine, galactose from uridine and rhamnose from thymidine (Robbins, Wright and Bellows, 1964).

GLYCOSYL TRANSFER AND SYNTHESIS OF POLYSACCHARIDES.

The glycosyl moieties are transferred from the sugar nucleotides to an acceptor during biosynthesis forming new glycosides. The transfer may lead to the formation of either α- or β- glycoside depending on the nature of the transferring enzyme.

The generalised reaction is :

$$\text{XDP - sugar} + \text{acceptor} \longrightarrow \text{sugar - acceptor} + \text{XDP}.$$

Theoretically it is reversible but the equilibrium is far right as large amounts of energy are available from hydrolysis of the nucleotide phosphate bond (Leloir, Cardini and Cabib, 1960). For example in the reaction

$$\text{UDPG} + \text{fructose} \longrightarrow \text{sucrose} + \text{UDP}$$

$$K_{eq} = \frac{[\text{sucrose}][\text{UDP}]}{[\text{UDPG}][\text{fructose}]} = 3{,}000 \qquad \text{(Mendicino, 1960)}$$

Also the formation of nucleotide sugars is exergonic due to breaking of the pyrophosphate bond of the nucleotide triphosphate (Kornberg, 1962).

There are now many known examples of glycosyl transfer leading to synthesis of new glycosidic compounds. A single sugar unit is transferred in the formation of trehalose phosphate (Leloir and Cabib, 1953) and sucrose phosphate (Cardini, Leloir and Chiriboga, 1955). Formation of other disaccharides and trisaccharides such as raffinose (Bourne, Walter and Pridham, 1965) also involve glycosidation of a monosaccharide or disaccharide by a sugar nucleotide.

Studies of the transferase enyzmes have proved difficult due to the crude symtems generally involved and their particulate nature. Attempts to solubilize them have generally resulted in inactivation. As far as may be ascertained, probably the transferases are quite specific towards the acceptor, the glycosyl moiety transferred and the nucleotide carrier. Thus certain mutants of E.coli are found to lack the enzyme UDPG pyrophosphorylase and although dTDPG is formed, it will not substitute for UDPG, and the cell wall lacks the glucose residue (Sundararajan, Rapin and Kalckar, 1962). Specificity with regard to the sugar moiety is indicated by a mutant of Salmonella typhimurium lacking the enzyme UDPGal-4-epimerase. It will incorporate galactose from UDPGal into the incomplete lipopolysaccharide but not glucose from UDPG (Rothfield, Osborn and Horecker, 1964). The question of specificity towards the acceptor molecule is most difficult to ascertain as the acceptor is often endogenous in the systems employed. It is almost impossible to separate enzyme from residual polysaccharide material. One example where specificity towards the acceptor has been shown is in the synthesis of starch by various plants. In spinach the primers are maltodextrins (Ghosh and Preiss, 1965) but in sweet corn, glycogen is required, maltodextrins, amylose or starch being inactive (Frydman and Cardini, 1964).

Synthesis of homopolysaccharides was assumed to occur in much the same way as had been shown for di- and trisaccharides, merely involving stepwise addition of more units. A primer was thought to be required, often a low molecular weight oligosaccharide of the polysaccharide. This would act as acceptor for the glycosyl units. Synthesis of cellulose from UDPG by a particulate enzyme preparation of Acetobacter xylinum occurred in the presence of soluble cellodextrins as primers (Glaser, 1958). During synthesis of glycogen in bacteria from ADPG, a primer of amylopectin, glycogen or dextrins was required (Kindt and Conrad, 1967). Recently de novo synthesis of glycogen has been reported in Klebsiella aerogenes. This raises the question of the nature of the first glycosyl transfer in this system, and whether the nucleotide sugar itself acts as acceptor (Gahan and Conrad, 1968).

One of the simplest heteropolysaccharides is hyaluronic acid, composed of a repeating disaccharide unit, N-acetylglucosamine and glucuronic acid. The glycosyl donors are UDPNAcglc and UDPGlUA, and a primer of performed hyaluronic acid or oligosaccharide may be required. The mechanism of synthesis is not known; there appear to be two alternatives (Markovitz, Cifonelli and Dorfman, 1959). There may be stepwise transfer of sugar units in turn from UDPNAcglc and UDPGlUA on to a primer using a single enzyme transferase with more than one active site. Alternatively there may be intermediate formation of a disaccharide which would then be transferred as such to the acceptor :

$$\text{UDPNAcglc} + \text{UDPGlUA} \longrightarrow \text{NAcglc} - \text{GlUA} - \text{UDP} + \text{UDP}.$$

No evidence has been found for such an intermediate. However the possibility of the disaccharide being linked to some carrier other than the nucleotide

was not investigated.

The type III Pneumococcus polysaccharide also consists of a repeating disaccharide unit:-

$$\left[3\beta\text{-D-glUA} - (1 \rightarrow 4) - \beta\text{-D-glc-1} \right]_n$$

The glycosyl donors in its synthesis are UDPG AND UDPGlUA. Again whether polymerization occurred by stepwise addition of monosaccharides or disaccharide units was not clear. There was no evidence for a UDP disaccharide (Mills and Smith, 1965), although again the possibility of linkage to other carriers was not fully considered. Endogenous polysaccharide may be removed from the system by the use of a specific depolymerase and primer requirements demonstrated. An oligosaccharide of n = 8 - 12 repeating units was required (Mills and Smith, 1962).

Recently a number of mucleotide-linked oligosaccharides have been isolated which were immediately implicated as possible intermediates in biosynthesis of polysaccharides. For example, in goat colostrum a nucleotide trisaccharide, UDPNAcglc-Gal-NAcneuraminic, was found. It was speculated that this may be involved in synthesis of mixed polymers like glycoprotein, glycolipids and also polysaccharides (Jourdain, Shimizu and Roseman, 1961). The trisaccharide is known to be present in the side chain of two glycoproteins (Spiro, 1962), so preformed oligosaccharides attached to nucleotides may be polymerized in formation of these. However, in other systems it seems likely that transfer of sugars occurs singly and this may be exemplified by the synthesis of chondroitin sulphate (Telser, Robinson and Dorfmann, 1966). Addition of NAcglc from UDPNAcglc occurred only when GlUA was at the non-reducing end of the oligosaccharide acceptor. Similarily addition of

GlUA from UDPGlUA occurred only when NAcglc was at the non-reducing end. This excludes the possibility of an intermediate disaccharide.

The synthesis of polysaccharides consisting of more than two monosaccharides is likely to be complex. At first heteropolysaccharides were thought to be much branched structures with no defined repeating unit (Aspinall, Jamieson and Wilkinson, 1956). It was proposed that, as there are so many specific heteropolymers and as the sugars to be transferred are components of nucleotides, a template may be required analegous to that used in synthesis of proteins from amino acids. This would ensure accuracy in the sequence of monosaccharides within the polymer. (Wilkinson, 1958). Furthermore it was known that the ability to synthesize a particular polysaccharide may be transmitted by DNA in transformation experiments, and that the composition of the polysaccharide was not affected by the levels of the nucleotide sugars involved in the synthesis. Alternatively formation of polysaccharide may be specified by the enzymes involved in its synthesis. The nuclear material would then be involved only in production of these enzymes (Watkiss and Morgan, 1959). Mutation at any locus coding for the enzymes would lead to loss of polysaccharide production and this has been demonstrated for a number of enzymes. In addition, with improved techniques of structural analysis, a small regular repeating unit has been found in most heteropolysaccharides in place of the complex structure envisaged previously. The number of specific enzymes required to synthesize the polymer is thus much reduced. At present then a template mechanism seems to be unnecessary.

The enzymes in different organisms vary widely. Loss or addition by recombination would result in a change in polysaccharide formed. This may

TABLE 1. (Contd.).

Sugar nucleotide	Polysaccharide	Ref.
TDPRha	into cell wall of Streptococcus	12
UDPNAcglc	into hyaluronic acid	13)
UDPGlUA	into hyaluronic acid	14)
UDPGal		15
GDPMan	into PS of K. aerogenes	15
UDPGlUA		15

HOMOPOLYSACCHARIDES

CMPNAcneuraminic	Colominic acid	16
UDPG	Cellulose	17
ADPG	glycogen	18
GDPM	mannan	19

TABLE 1: SYNTHESIS OF POLYSACCHARIDES FROM SUGAR NUCLEOTIDES BY PARTICULATE BACTERIAL PREPARATIONS.

Sugar nucleotide	Polysaccharide	Ref.
HETEROPOLYSACCHARIDES		
CMPKDO		1
UDPG		2
UDPGal		2
UDPNAcglc	into LPS of *Salmonella* and *E.coli*	2
GDPM		3
GDPCol		1
dTDPRha		3
CDPAbe		4
UDPNAcmuramic acid peptide	into murein	5
UDPNAcglc		6
UDPG		7
UDPNAcglc	into teichoic acids	8
CDPribitol		9
CDPglycerol		10
UDPNAcglc		11
UDPGalUA		11
UDPG	into PS of Pneumococcus	11
UDPGlUA		11
UDPGal		11

be seen, for example, by loss of pyrophosphorylase activity in a mutant of S.typhimurium lacking UDPG pyrophosphorylase. An incomplete lipopolysaccharide was produced without glucose and the O-specific antigenic determinants. There may be modification of the polysaccharide as in phage conversion by ϵ^{15} which, in S. anatum, caused two structural changes in the O-Antigen by forming β-galactose links instead of α- and by eliminating O-acetyl groups (Robbins and Uchida, 1965). There may be alteration in one of the components to which the polysaccharide is attached, either another polysaccharide as in the Ra mutants of Salmonella (Stocker, Wilkinson and Mäkelä, 1966), or in complex structures where the polysaccharide is attached say to teichoic acid, lipid or protein. In addition there is the possibility that the structure of the polysaccharide may be determined by the relative activities of the enzymes synthesizing it. In S. aureus the repeating unit of the teichoic acid polymer is 4 α- or β- NAcglucosaminyl-ribitol linked by 1, 5 - phosphodiester bridges. The proportion of α- and β- linkages was found to vary with different strains. This in turn altered the serological determinants so that differences in α- and β- transferase activities affected the end products (Nathenson and Strominger, 1962).

Table 1 lists particulate systems where synthesis of polysaccharides has been accomplished from sugar nucleotides.

Assuming a repeating unit in the heteropolysaccharide, synthesis could occur in several possible ways :

I. There may be sequential addition of activated monosaccharides to an acceptor as with homopolysaccharide synthesis.

II. There may be addition of an activated monosaccharide to another

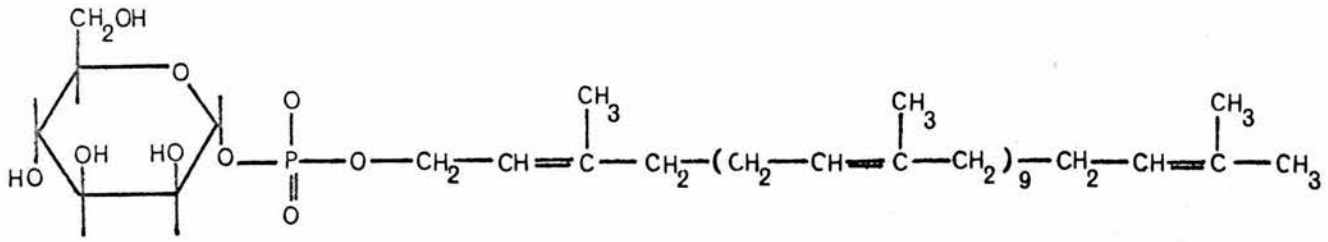


FIG 4 Mannosyl Lipid Intermediate

to form an activated disaccharide, then addition of another to form an activated trisaccharide which may be added to an acceptor, or polymerized first before addition.

III There may be sequential addition of activated monosaccharides to some intermediate carrier which are then added as a unit to the acceptor, or polymerized first before addition.

A review published in 1964 on the lipopolysaccharides of the Gram-negative cell wall states that the O-antigen chains of S.typhimurium consist of the repeating unit gal-man-rha and "considerable interest has been expressed in the possibility that the repeating unit might be synthesized as a nucleotide oligosaccharide and transferred into the growing polysaccharide as a unit" (Osborn et al 1964). Since then Scheme III has been definitely demonstrated in the synthesis of O-antigens in Salmonella species and has also been shown for a number of other systems. These include the mannan synthesized by Micrococcus lysodeikticus (Lennarz, 1964), the murein synthesized as a cell wall component (see Section C), and perhaps cellulose synthesized extracellularily by A. xylinum (see Section B).

The intermediate carrier has been shown to be lipid in nature. In all but the cellulose system where it has not been characterized, it is a C_{55} polyisoprenyl compound. It is linked to the sugar via a phosphodiester bridge in the case of mannan (Scher, Lennarz and Sweeley, 1968) and via a pyrophosphate ester link in the case of O-Antigen and murein, one phosphate being derived from the nucleotide and one from the lipid (Higashi, Strominger and Sweeley, 1967). The mannosyl lipid intermediate is illustrated in Fig.4. It is interesting that these lipids may function as carriers in synthesis of homopolysaccharides, in addition to complex heteropolysaccharides. In

synthesis of other homopolysaccharides, lipid intermediates may be involved which have not so far been detected, although in a recent paper describing de novo synthesis of glycogen no sign of such an intermediate was found (Gahan and Conrad, 1968).

The structure and biosynthesis of the extracellular polysaccharides, the murein of the cell wall, the lipopolysaccharide of the Gram-negative cell wall and the intracellular polysaccharide glycogen will now be considered in greater detail.

INTRODUCTION

SECTION B

EXTRACELLULAR POLYSACCHARIDES

Research into the nature of bacterial polysaccharides was stimulated by the work of Heidelberger's group (Heidelberger and Avery, 1923; Heidelberger, Goebel and Avery, 1925) which demonstrated the carbohydrate nature of the "specific soluble substance" of Pneumococcus and its responsibility for type specificity. The connection between serological specificity and chemical structure was realized. At the same time strains of Freidlander's bacillus (K. pneumoniae) were shown to be of three capsular types (Toenniessen, 1921). These could be classified serologically (Julianelle, 1926) and were chemically distinct (Goebel and Avery, 1927). A number of pathogenic organisms produce extracellular material when first isolated, particularly species of Enterobacteriaceae and Pneumococcus. Most work has been done using these strains due to their medical importance.

STAINING

The development of extracellular polysaccharides may be dependent on the environmental conditions, and the amount on the relative availability of nutritional factors and the stage of growth. Many methods have been developed to show capsules and slime in the light microscope. Capsules have little affinity for normal dyes and are best shown by "negative staining" in which they are outlined against a dark background. India ink is commonly used (Duguid, 1951). As the capsules contain up to 98% water, distortion due to dehydration will occur if the preparation is dried. For this reason capsules cannot generally be seen satisfactorily in the electron microscope. The capsule may be outlined by combination with the homologous antibody. This

is a specific reaction and is used in serological typing. Sometimes the capsule may be so thin as to be undetectable in the light microscope. Serological or chemical methods are used to distinguish it, for example the V_i antigen of S. typhi. Sometimes it is difficult to separate cell wall and capsular polymers. This is exemplified by the O-antigenic polysaccharides of Salmonella which were termed "microcapsules" (Wilkinson, 1958). They were later shown to be part of the lipopolysaccharide of the cell wall.

As might be expected, little internal structure has been revealed within the capsule. However, in E. coli Lisbonne strain, striated fibres were demonstrated at right angles to the cell surface running through the amorphous capsular material (Labaw and Mosley, 1954). In B.megaterium bands of polysaccharide material were shown to occur within the polypeptide capsule, by use of specific antisera (Tomcsik, 1951).

FUNCTION

The importance of extracellular polysaccharides in determining virulence of pathogenic bacteria has been recognized since 1927 when Smith isolated E. coli from a case of white scours in calves. He separated capsulated and non-capsulated mutants by colony appearance and found the latter were less virulent, more agglutinable and were taken up better by leucocytes than the former. In the laboratory bacteria are found to survive just as well without a capsule. The capsule may be artificially stripped from the cell by the use of a specific depolymerase as in the decomposition of capsular polysaccharide of Pneumococcus Type III by a micro-organism from soil (Dubos and Avery, 1931) This does not affect the viability of the cell.

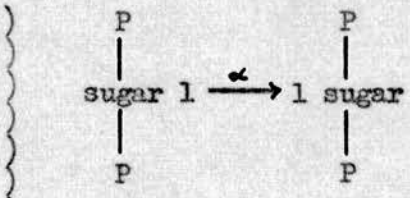
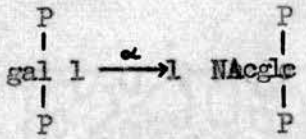
In natural environments, however, a capsule may be important in survival. It does not act as a storage compound, the cell being generally unable to utilize it, once formed. The ability to produce it may be lost by a single mutation.

The capsule confers a negative charge on the organism due to phosphate groups, sialic acids, hexuronic acids or N-acetyl aminohexuronic acids. In pathogenic organisms this charge may render the bacteria less liable to phagocytosis (Enders, Shaffer and Wu, 1936). There may be protection of the surface from complement and antibody, and perhaps non-specific inhibition by combination of the polysaccharide with antibacterial substances in the blood such as properdin and lysozyme (Skarnes and Watson, 1955; Pillemer, Schoenberg, Blum and Wurtz, 1955). In saprophytes there may be similar protection against engulfment by protozoa (Singh, 1942). In a natural environment there may be changing conditions of drought followed by rapid exposure to water. Mucoid strains are found to survive drying better than non-mucoid (Morgan and Beckwith, 1939) and the capsule may thus act as a buffer against dessication. In a similar way it may prevent water being gained too rapidly after drying (Wilkinson, 1958). It has been shown in dried yeasts that repair of the membrane on rehydration is more efficient if water is allowed to reach the cell wall slowly (Herrera, Peterson, Cooper and Peppler, 1956). The capsule may protect the bacterium against attack by bacteriophage (Kauffmann and Vahlne, 1945) as often the receptor sites for these are on the cell wall, although the capsule itself may be the substrate for phage-induced depolymerases (Adams and Park, 1956). Other functions proposed are to aid dispersal due to the charge conferred by the component sugars, or to aid uptake of ions due to their absorption by the polysaccharide (Rorem, 1955)

Organism	Serotype	Sugar composition of PS	Reference	Structural details	Reference
Gram-negative soil bacterium	-	D-rha, D-talomethylose	68,69	-	
Unidentified coccus	-	NAcglc, glUA	70	NAcglc$_{\beta}$1 ⟶ 3 glUA$_{\beta}$1 ⟶ 4 repeating unit	70

Organism	Serotype	Sugar composition of PS	Reference	Structural details	Reference
Serratia marcescens	-	a) glUA, glc, man b) rha, glc c) D-glycero-D-mannoheptose L-glycero-D-mannoheptose d) rha, glc, heptose	54 55	-	
Pasteurella multocida	-	fructose, glc, man, $glcNH_2$	56	-	
Azotobacter vinelandii	-	galUA, manUA, rha, O-Ac, 2-keto-3-deoxygalUA	57,58	-	
A. chroococcum	-	glc	59	-	
A. azilis	-	gal, rha, 2-keto-3-deoxygalUA	60	-	
Arthrobacter viscosus	-	gal, glc, manUA, O-Ac	61	-	
A. globiformis	-	glUA, glc	62	-	
Streptococcus bovis	-	glc	63	β(2 ⟶ 6) linkages	63
Xanthemonas campestris	-	glc, man, glUA, acetyl, pyruvate	64	-	
Sarcina ?	-	man	65	(1⟶2), (1⟶3), (1⟶6) linkages	65
Alcaligenes faecalis var. myxogenes	-	glc, gal, man, pyruvate	66	-	
Agrobacter radiobacter	-	glc	53	-	
A. tumefaciens	-	glc	53	-	
Mycobacterium tuberculosis	-	man, glc, P	67	-	

Organism	Serotype	Sugar composition of PS	Reference	Structural details	Reference
Neisseria meningitidis	-	NAcneuraminic, $glcNH_2$	43	-	
Pseudomonas aeruginosa	-	fuc, glc, gal, $glcNH_2$, $galNH_2$ hexosamine	44,45	-	
	-	fuc, glc, gal, $glcNH_2$, $galNH_2$ NAcneuraminic	44,45	-	
	-	manUA, galUA, O-Ac	46	-	
	-	glUA, gal, man, rha	47	-	
L.mesenteroides and many others grown on sucrose	-	glc	48	$\alpha(1 \longrightarrow 6)$ linkages	48
Zymomonas mobilis, and Aerobacter hevanicum and others grown on sucrose	-	fructose	48,49	$\beta(2 \longrightarrow 6)$ linkages	48,49
Sphaerotilis natans	-	glc, gal, glUA, fuc	50	-	
Citrobacter freundi	-	NAcneuramic, NAcglc, NAcfuc	51	-	
Rhizobium meliloti	-	glc, gal, glUA	52	-	
R.leguminosarium	-	glc, glUA	47,53	-	
R.trifolii	-	glc, glUA, 4methoxy-glUA	47	-	
R.phaseoli	-	glc, glUA, 4methoxy-glUA	47,53	-	
R.japonicum	-	glc, rha	53	-	

Organism	Serotype	Sugar composition of PS	Reference	Structural details	Reference
	XIA	glc,gal,glycerol,P,O-Ac	30	-	
	XII	glc,gal,amino sugar, O-Ac	30	-	
	XIII	glc,gal,$glcNH_2$,ribitol P,O-Ac	30	-	
Haemophilus Influenzae	a	glc,P	36		35, 36
	b	ribose, P	34,35		
	c	hexose, P	34		
	f	$galNH_2$,P	35		
	d	NAcglc,NAcgalUA	37		
	e	hexose (L-glucose?),$glcNH_2$	35		
S.typhi and a few other Enterobacteriaceae	V	NAcgalUA,O-Ac	38,40	(1 ⟶ 4) linkages	39
Vibrio ?		hexosamine,O-Ac	41	-	
Haemophilus suis	-	NAcglc,gal,P	37		37, 42

Organism	Serotype	Sugar composition of PS	Reference	Structural details	Reference
	XVI	gal,glc,glcNH$_2$,rha,glycerol, P, acetyl	30	-	
	XIX	glc,gal,glcNH$_2$,ribitol P, pentose, O-Ac	30	-	
	XX	glc,gal,glcNH$_2$,ribitol P,O-Ac	30	-	
	XXI	glc,gal,glcNH$_2$,rha,P,O-Ac	30	-	
	XXVII	glc,gal,glcNH$_2$,uronic acid,rha, P, O-Ac	30	-	
	XXX	glc,gal,galNH$_2$,ribitol P,O-Ac	30	-	
	XVIII	glc,gal,rha,glycerol,P,ribitol	31	gal 1⟶4 glc 1⟶6 glc 1⟶3 rha 1⟶4 glc 3	
	XXXIV	galactofuranose,glc,ribitol P	30,32	galfuran 1⟶5 glc 1⟶2 galfuran 1⟶5 gal (1⟶2 ribitol - P)	30, 32
	IV	gal,amino sugar, O-Ac	33	-	
	X	gal,glcNH$_2$,ribitol P, O-Ac	30	-	
	XA	galactofuranose,galNH$_2$,ribitol P	30	-	
	XI	gal,glc,glcNH$_2$,galNH$_2$,ribitol P O-Ac	30	-	

Organism	Serotype	Sugar composition of PS	Reference	Structural details	Reference
	VIII	gal, glc, glUA	22	glUA$_{\beta}$ 1 ⟶ 4 glc$_{\beta}$ 1⟶ 4 gal repeating unit	22
	V	glc, glUA, fucNH$_2$, 2NAc2, 6-dideoxytalose	23	glUA$_{\beta}$ 1⟶3 fucNH$_2$$_{\beta}$ 1 ⟶4 glc	24
	VI	gal, glc, rha, ribitol, P	25,26	gal 1⟶ 3 glc 1⟶3 rha 1⟶3 ribitol (1 → 2 P) repeating unit	25, 26
	VII	gal, glc, rha, galNH$_2$	27	-	
	IX	glc, glUA, glcNH$_2$, NAcglc	24	-	
	XII	glc, glUA, glcNH$_2$, gal	24	-	
	XIV	NAcglc, gal, glc	28,29	gal (1 → 6) gal 1 ⟶ 4 NAcglc 1 ⟶ 4 gal (1 → 6) glc 1 ⟶ 4 NAcglc (1 → 4) glc	28, 29
	XV	gal, glcNH$_2$, glc, ribitol P, galNH$_2$, O-Ac, glycerol	30	-	
	XVA	gal, glc, glcNH$_2$, ribitol P glycerol, O-Ac	30	-	
	XVB	gal, glc, glcNH$_2$, ribitol P glycerol, O-Ac	30	-	

Organism	Serotype	Sugar composition of PS	Reference	Structural details	Reference
	?	man, glc, gal, glUA	14	glc–man–glc–man | glc | man | glUA	14
	K28	gal, glc, man, hexNH_2, fuc	13	-	
	K34	gal, glc, man, hexNH_2	13	-	
	K42	galUA, gal, fuc, O-Ac	10,11,15	gal 1 ⟶ 3 galUA 1 ⟶ 2 fuc repeating unit	15
	K85	glUA, man, NAcglc, rha	10,11,16	glUA 1 ⟶ 2 man 1 ⟶ 3 man 1 ⟶ 3 NAcglc rha rha	16
	K 1	NAcneuraminic	2	-	
	?	NAcneuraminic, glcNH_2, gal, glc	2	-	
	?	NAcneuraminic, gal, galNH_2	17	-	
	K 7	manNH_2, glcNH_2, glc, gal	2	-	
Pneumococcus	I	gal, fuc, NAcglc, galUA, OAc	18	-	
	II	glUA, glc, rha	19		
	III	glc, glUA	20	glcUA$_\beta$ 1 ⟶ 4 glc$_\beta$ 1 ⟶ 3 glc$_\beta$ 1 ⟶ 4 glUA repeating unit	21

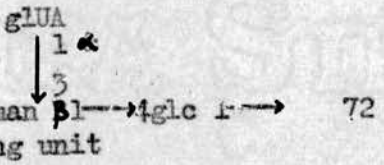
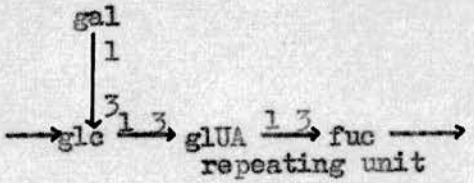
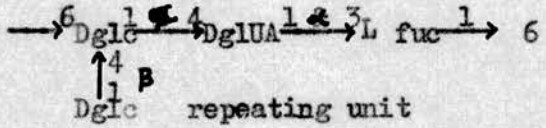
Organism	Serotype	Sugar composition of PS.	Reference	Structural details	Reference
	NCTC 418 Oxford strain	glUA, glc, man, manUA	9	(glUA 1 → 4 man) and (manUA 1 → 4 glc) linkages	9
	NCTC 243 NCTC 418 NCTC 5055 NCTC 9504	glc, man, glUA	72		72
E.coli	K27	glUA, gal, glc, O-Ac, fuc	10,11,71		71
	K30	glUA, gal, man, fuc	10,11	man 1 → 2 glUA 1 → 3 gal	12
	K87	glUA, $glcNH_2$, gal glc, rha	10,11	-	
	K4	galUA, $galNH_2$, gal, glc	10,11	-	
	K8	glUA, $galNH_2$, $glcNH_2$, gal, man	10,11	-	
	K34	gal, glc, man	13	-	
	K17	glUA, $glcNH_2$, gal, glc	10,11	-	
	?	$glcNH_2$, gal, rha, glycerol, P	2	-	
	?	NAcneuraminic	2	NAcneuraminic 1 → 8 NAcneuraminic	2

TABLE 2.

MONOSACCHARIDE UNITS AND STRUCTURE OF EXOPOLYSACCHARIDES OF BACTERIA

Organism	Serotype	Sugar composition of PS.	Reference	Structural details	Reference
Klebsiella	1	glUA,gal,glc,fuc,pyruvic	1, 2	-	
	2	glUA,glc,rha,pyruvic	1, 2	-	
	3	galUA,man,gal, pyruvic	1, 2, 3	(galUA⟶man) and (gal⟶4 man) linkages	3
	4	gal,glc,man,pyruvic	1, 2	-	
	5	gal,glc,man,pyruvic	2		
	8	gal,glc	4		
	26	gal,glc,man	4		
	29	gal,man	4		
	54	glUA,fuc,glc	5	 repeating unit	6
	57	gal,man	4	-	
	64	glUA,glc,man,rha	7	-	
	11	gal,glc,man	8	-	
	21	gal,man	8	-	

STRUCTURE

In Table 2 are listed the monosaccharides found in the extracellular polysaccharides of a variety of organisms together with any structural details known. Serological relationships and chemical structure have not been correlated in most instances except in Pneumococcus, E.coli and Klebsiella groups. This information may be of value in taxonomy as in Rhizobium (Graham, 1965).

Determination of structure is difficult especially as the chemical methods of methylation and partial acid hydrolysis led to the concept of polysaccharides being highly branched complex structures (Aspinall, Jamieson and Wilkinson, 1956), possibly due to incomplete methylation. Since then with the improvement in techniques, this view has been altered. Polysaccharides are generally thought to consist of simple repeating units.

Two main methods are employed to determine order and linkage of monosaccharide units, their branch points and whether the sequence is repeating.

The first is partial acid hydrolysis. Using different acids and conditions varying products are formed depending on the acid lability of the linkages. Neutral disaccharides such as maltose are fairly acid labile; if one component is a hexosamine or a uronic acid, more severe conditions are required. The products may be separated by ion exchange columns or chromatography and component sugars found by further hydrolysis.

The second is enzyme hydrolysis which is generally more specific. Some micro-organisms possess enzymes decomposing capsular polysaccharides. General elective culture techniques are used to find these. For example

a Bacillus palustris strain was isolated (Torriani and Pappenheimer, 1962) which would grow on Type III Pneumococcal capsular material. The products of enzyme hydrolysis were examined by chromatography and the enzymes were thought to cleave linkages between β-glucuronosyl (1→4) glucose at random along the chain.

An alternative to this method is provided by the use of depolymerases obtained from phage-infected bacteria. Adams and Park (1956) found such a system in phage infected cells of K.pneumoniae Type 2. These liberated a depolymerase capable of hydrolyzing the capsule of the organism. Similar phages in strains of E.coli and K. aerogenes (Sutherland and Wilkinson, 1965) have been discovered.

One further enzymic technique used is sequential enzyme induction. The sequence of enzyme induction in a particular organism can be predicted, as the enzyme system required to decompose a component is not induced until the other components are preferentially exhausted. Barker, Pardoe, Stacey and Hopton (1963) used B. palustris which was incubated with orosomucoid, a complex mucoprotein. There was sequential release of N-acetyl neuraminic acid, N-acetyl mannosamine, galactose and an oligosaccharide. As the products cannot be utilized by the organism, they accumulate in the medium and the arrangement of sugar molecules may be deduced. They also used a strain of K. aerogenes in the same manner to degrade the capsular polysaccharide of Pneumococcus Type XIV (Barker et al, 1964).

Sereological methods are used to aid in determination of structure as it has been found that polysaccharides are often determinant molecules for the immunological specificity of bacteria. Cross reactions may determine or verify structure. If a polysaccharide precipitates with the heterologous

antibody then it must possess some linkage and components in common with that antigen. For example, Azotobacter chroococcum cross-reacts with anti-Pneumococcus Type III serum which can be attributed to common cellobiuronic acid residues (Cooper, Baker and Stacey, 1938).

Knowledge about the nature of linkage within a small unit can be obtained from optical rotation measurement and its change on hydrolysis of the unit or by use of specific enzymes. The infra-red spectrum may also give some information. The point of linkage is most often obtained from methylation and identification of the methyl sugars after hydrolysis, or by periodate oxidation.

Examples illustrating these methods are taken from Pneumococcus and K. aerogenes strains.

In the genus Pneumococcus over eighty different capsular types are known. Type III is one of the simplest being composed of two sugars, glucose and glucuronic acid. It was highly purified (Heidelberger, Goebel and Avery, 1925) and on hydrolysis an aldobiuronic acid was released, identified as cellobiuronic acid from its methylation derivations (Hotchiss and Goebel, 1937). The polysaccharide was further analysed chemically (Reeves and Goebel, 1941) and the following structure proposed :

$$\left[3\,\beta\text{-D-glUA-}(1\longrightarrow4)\text{-}\beta\text{-D-glc-1}\right]_n$$

The polysaccharide was degraded by enzymes produced by other organisms (Sickles and Shaw, 1934). Its antiserum cross reacted with the polysaccharide of Rhizobium radicicolum and A. chroococcum (Lawson and Stacey, 1954) due to the presence of cellobiuronic acid residues in all three polymers.

The repeating unit for Type VIII Polysaccharide has been worked out in

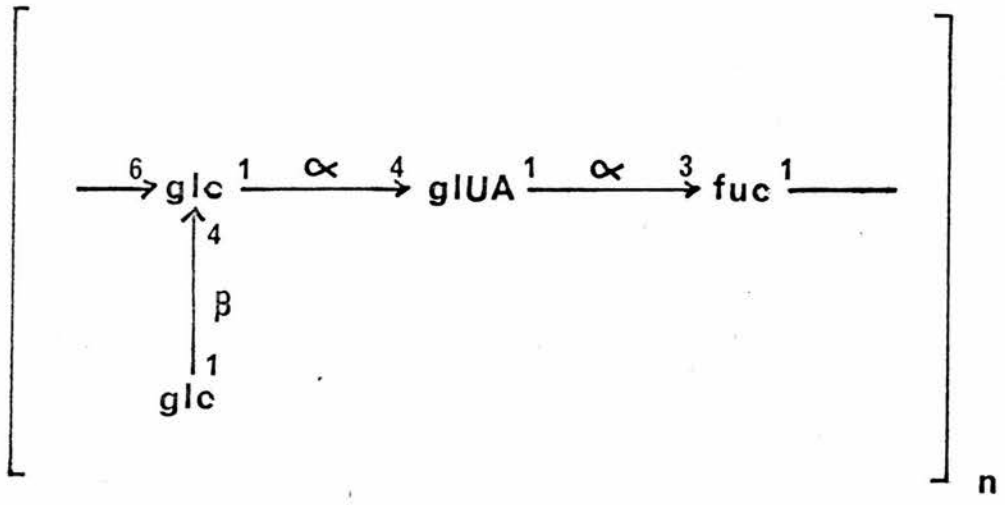


FIG 5. REPEATING UNIT OF A3 POLYSACCHARIDE

detail (Jones and Perry, 1957). It is more complex than Type III being composed of a tetrasaccharide :

$$-\left[4-\beta\text{-glUA}\ (1\rightarrow4)\,\beta\text{-glc}\ (1\rightarrow4)\,\alpha\text{-glc}\ (1\rightarrow4)\,\alpha\text{-gal}\ 1\right]_n$$

Parts of the structures of other types have also been found and the chemical basis for some serological cross reactions discovered including the blood group substances which cross react with Type XIV antiserum (Finland and Curnen, 1938; Barker, Keith and Stacey, 1961).

All naturally occurring strains of Klebsiella are mucoid and capsulate and a large number of types have been discovered. Kauffmann (1951) published the first antigenic classification of Klebsiella based on recognition of somatic (O) and capsular (K) antigens. Edwards and Fife (1952) extended this work to include 57 immunologically distinct K types.

K. aerogenes Type 54 strain A3 is a heteropolysaccharide composed of glucose, fucose, glucuronic acid in ration 2:1:1 (Duguid and Wilkinson, 1953). Early structural studies by partial acid hydrolysis and methylation had indicated a complex highly branched structure (Aspinall, Jamieson and Wilkinson, 1956). Sandford and Conrad (1966) using improved methylation techniques suggested the possibility of the polysaccharide being composed of a simple repeating unit. Acid hydrolysis yielded cellobiose, glucuronosyl-fucose, a trisaccharide containing an additional glucose residue, and a tetrasaccharide with two additional glucose residues. The repeating unit they proposed is shown in Fig.5. However, using phage-induced depolymerases, hydrolysis of the polysaccharide to oligosaccharides similar to the tetrasaccharide above, but not identical, was obtained. Sutherland (1967) concluded that O-acetyl groups were present plus another labile group, and that a repeating unit of

eight sugars was more likely. The O-acetyl groups would be lost on acid hydrolysis.

SYNTHESIS

CULTURAL CONDITIONS

Synthesis of extracellular polysaccharide in a particular organism leads to the colony form being mucoid with a slimy or gelatinous appearance. In liquid media the viscosity may be greatly increased particularly in the case of a slime-producer. A. xylinum produces a thick pellicle of cellulose fibres in the growth medium (Brown, 1886), and dextran is produced as a jelly-like mass which has been known since the middle of the last century in the wine and sugar beet industries.

There are cultural conditions in which polysaccharide is produced in large quantities and others in which it is hardly produced at all. The influence of these environmental conditions was studied in K. aerogenes Type 54 (Duguid and Wilkinson, 1953). When growth was limited by the carbon and energy source, little polysaccharide was formed. On the other hand, when nitrogen was limiting, polysaccharide production rose to a maximum. At limiting levels of sulphur or phosphorus it also rose to a maximum. K^+ and preferably Mg^{++} ions must be present. This indicates that N, S or P may not be required in the synthesis of polysaccharide and, acting as growth limiting factors, may channel the metabolic activities of the cell into its production, utilizing the source of carbon and energy.

Washed cell suspensions of K. aerogenes Type 54 were used to examine the effects of various nutrients on polysaccharide production (Wilkinson and Stark, 1956). In the presence of glucose, intracellular and extracellular

polysaccharide was formed and O_2 consumption corresponded to 65% of that required for complete oxidation of glucose. Of the 35% assimilated, about 18% is accounted for by the production of polysaccharide. K^+ ions were again required and Mg^{++} to a smaller extent. Ca^{++} ions stimulated extracellular polysaccharide production and phosphate ions were inhibiting. Anaerobiosis decreased the rate of polysaccharide synthesis, this being related to the amount of energy derived from aerobic and anaerobic conditions. Temperature may have a significant effect although this was not marked in K. aerogenes. Generally lowered incubation temperature causes increased polysaccharide formation.

The carbon source may influence polysaccharide production in different ways. Some bacteria only form slime when growing at the expense of a specific carbon source required as substrate. Thus L.mesenteroides only produces dextran at the expense of sucrose; neither glucose nor fructose act as substrates. Using E.coli, polysaccharide production was measured after growth on various substrates (Siegel and Clifton, 1950). This was found to be related, not to the free energy of exidation, but to the suitability of the intermediates. The same polysaccharide is produced on all substrates (Wilkinson, Dudman and Aspinall, 1955).

CELL-FREE SYNTHESIS

Cell-free systems for synthesis of extracellular polysaccharides have been successful only in a few instances.

As has been mentioned in Section A, the synthesis of dextran and levan from sucrose using cell-free filtrates containing dextransucrose and levan-sucrose was accomplished before anything was known about the precursors of polysaccharides. Dextransucrose transfers glucose units from sucrose to a

wide variety of receptor molecules including maltose without the need for phosphorylated intermediates, (Bailey, Barker, Bourne and Stacey, 1955). Where sucrose only is present, it may act as both acceptor and substrate, so that a trisaccharide may be formed in the initial step of the polymerization (Barker, Bourne and Theander, 1957) :

glc (1⟶2) fru + glc (1⟶2) fru ⟶ glc (1⟶6) glc (1⟶2) fru + fru

The synthesis of cellulose by A. xylinum provided the first cell-free system utilizing a nucleotide sugar as precursor. Previously it had been shown that in the fungus Neurospora crassa synthesis of chitin occurred by glycosyl transfer from UDPNacetylglucosamine (Glaser and Brown, 1957). A particulate enzyme preparation was then obtained from A. xylinum which would synthesize cellulose from UDPG in the presence of cellodextrins as primer (Glaser, 1958). Endogenous cellulose could be removed by treatment with cellulase. This essentially abolished synthesis of cellulose unless cellodextrins were added. Attempts to solubilize the particulate enzyme failed although part could be obtained as a soluble system by extraction with digitonin and action of lipase. The solubilized enzyme was extremely labile. More than one enzyme was thought to be involved in synthesis and short chain cellodextrins may be produced by transglycosidation followed by formation of typical cellulose fibrils. This was assumed to occur by a process of crystallization and not by action of extracellular enzymes (Ben-Hayzin and Ohad, 1965). A possible precursor of cellodextrins was found (Khan and Colvin, 1961). It was a glucose-lipid complex which would form microfilaments of cellulose in aqueous solutions containing an extracellular enzyme. Bound-activated glucose may therefore be transported across the wall as a lipid

complex, then the glucose transferred to the acceptor while the lipid is recycled. This mechanism is analogous to that found for O-antigen, mannan and murein synthesis but not so far found in any other extracellular systems.

A particulate enzyme system from E.coli was used to synthesize colominic acid, a homopolymer of N-acetylneuraminic acid, from CMPacetylneuramic acid. Some colominic acid remained bound to the enzyme preparation and efforts to remove it also inactivated the enzyme (Aminoff, Dodyk and Roseman, 1963).

The majority of cell-free systems successfully synthesizing polysaccharide have been found in Pneumococcal species. Type III polysaccharide is composed of a repeating disaccharide unit :

$$\left[3\text{-}\beta\text{-glUA}\ (1\longrightarrow 4)\ \beta\text{-Dglc}\ 1\text{-}\right]_n$$

A particulate enzyme preparation sedimenting between 30,000 and 100,000g incorporated glucose and glucuronic acid from UDPG and UDPGlUA respectively into the polysaccharide fraction (Smith, Mills, Bernheimer and Austrian, 1960). Preformed polysaccharide may be removed by prior incubation with a depolymerase and net synthesis shown. The depolymerase acts as a randon endo-β-glucuronidase and, by allowing it to work for varying lengths of times, oligosaccharides of different lengths may be prepared. The primer requirement was for a repeating unit of $n = 8 - 12$. If $n = 1$, 2 or 4 there was no stimulation of synthesis. During formation of the polysaccharide, molecules of varying sized were formed early on and the chain length of these increased as the reaction proceeded. Lengthening may occur by stepwise addition of monosaccharide or disaccharide units preformed as UDPGlUA - Glc. However no evidence has been uncovered so far for such an intermediate. The enzyme preparation cannot be solubilized without complete loss of activity and may be an integral part of the

membrane (Mills and Smith, 1965).

Type I polysaccharide contains galactose, fucose, galacturonic acid and N-acetylglucosamine in addition to O-acetyl groups (Smith, Galloway and Mills, 1960). Its structure is not known although it may contain a backbone of polygalacturonic acid. A particulate enzyme fraction from a non-capsulate mutant lacking the enzyme UDP-glucuronic-4-epimerase formed a polymer from UDP-galacturonic acid reacting serologically with Type I antiserum (Smith, Galloway and Mills, 1961). Addition of UDPNAcetylglucosamine caused 100% increase in synthesis provided UDPGalUA was also present. It may be added to the polygalacturonic acid backbone as a side chain.

The polysaccharide of Type VIII contains the repeating unit

$$\left[4\ \beta\ \text{glUA}\ (1\rightarrow4)\ \beta\ \text{glc}\ (1\rightarrow4)\ \alpha\ \text{glc}\ (1\rightarrow4)\ \alpha\ \text{gal}\ 1\rightarrow\right]$$

A particulate preparation incorporated glucose from UDPG in the presence of UDPGal and UDPGlUA into the polysaccharide. The product was precipitable with Type VIII antiserum (Mills and Smith, 1962).

Type XIV polysaccharide consists of N-acetylglucosamine, galactose and glucose and its structure has been worked out in some detail, (Barker, Keith and Stacey, 1961; Barker, Heidelberger, Stacey and Tipper, 1958). A cell-free particulate preparation incorporated the three sugars into material which reacted with Type XIV antiserum. It was not identical with the native polysaccharide, however, as the component sugars were not present in the same proportions (Distler and Roseman, 1964). Synthesis of two glycolipids also occurred : α-D-glc (1—3) diglycerides and α-D-gal (1→2)α-D-glc (1→3) diglycerides (Kaufman, Kundig, Distler and Roseman, 1965). These are unlikely to be intermediates in the synthesis of Type XIV polysaccharide as the same diglycerides occurred in an R-mutant of Type I Pneumococcus (Brundish, Shaw and

Baddiley, 1965). Similar glycolipids are widespread in Pneumococcus and other groups such as the dimannosyl-diglyceride found in M.lysodeikticus (Lennarz, 1964). They may be intermediates in the synthesis of cell wall components.

The extracellular polysaccharide of one strain of K. aerogenes contains galactose, mannose and glucuronic acid. Studies made by Troy and Heath (1968) indicate that an oligosaccharide - lipid intermediate may be involved in synthesis of this polysaccharide. The cell envelope fraction of a mutant lacking the enzyme UDPGal - 4 - epimerase was incubated with labelled UDPGal. The radioactivity was incorporated into the chloroform-methanol soluble fraction and, when UDPGlUA and GDPMan were also added to the system, the radioactivity was incorporated into polysaccharide material. Addition of GDPMan without UDPGlUA resulted in the accumulation of a lipid material containing galactose and mannose in the ratio 1 : 1. It was thought that gal-1-Ⓟ may be added to the lipid acceptor first, with the release of UMP, followed by mannose to make an oligosaccharide - lipid intermediate.

CONTROL OF POLYSACCHARIDE SYNTHESIS

The ability to produce an extracellular polysaccharide is a stable genetic character. Little is known about the regulation of its production and whether the genes involved may be clustered in an operon, controlled by regulators. It is easy to obtain non-capsulate mutants from capsulate strains. Indeed these often arise spontaneously particularly in old cultures. However the reverse has never been reported, that is a non-capsulate cell regaining the ability to produce the polysaccharide.

In non-capsulate cells, generally there is no marked accumulation of

nucleotide precursors. Synthesis of these may therefore be under some type of negative feedback control. Pyrophosphorylases would be the most logical point for such control as they represent the first step in commitment of the monosaccharide to polysaccharide synthesis. In strains lacking the enzymes necessary to form the final polysaccharide, there is no alteration in pyrophosphorylase levels or levels of later enzymes. Feedback control at the level of enzyme action or in formation of earlier compounds such as glucose - 1 - Ⓟ or glucose - 6 - Ⓟ may occur. Several nucleotide sugars control their own rate of biosynthesis by feedback inhibition, and, if very slight increase in concentration of nucleotides occurs, this may be sufficient to inhibit synthesis of the precursors. Thus dTDP-rhamnose was found to inhibit dTDP-glc pyrophosphorylase (Bernstein and Robbins, 1965), and CDP-paratose to inhibit CDP-glc pyrophosphorylase (Mayer and Ginsburg, 1965). In S. champign which has a polysaccharide containing both mannose and fucose, GDPfucose inhibited GDP-mannose hydro-lase and GDPMan inhibited GDPMan pyrophosphorylase (Kornfeld and Ginsburg, 1966). This indicates independent control of the rate of synthesis of the nucleotide sugars acting as donors in polysaccharide synthesis.

The polysaccharide may contain modifying groups in addition to monosaccharide components, such as acetyl, for example in K. aerogenes A3 (Sutherland, 1967), pyruvyl, for example in Xanthemonas campestris (Sloneker and Orentas, 1962), or phosphate, for example in Pneumococcus Type VI (Rebers and Heidelberger, 1959; 1961). These increase the variety of antigenic determinants. They may also be concerned with the control of polysaccharide production by their relative availability above that required for the general metabolism of the cell (I.W. Sutherland, unpublished).

Transformation of the capsule type has been achieved in Pneumococcus, Haemophilis influenzae and Neisseria meningitidis. Griffith in 1928 demonstrated transformation in Pneumococcus from an R-variant to an S-type. Later capsule formation was found to be induced in an R-variant using DNA from a heterologous capsulated type (Avery, MacLeod and McCarty, 1944). Transformation with DNA from Type I cells into non-capsulated Type III with formation of capsulated Type I transformants has been shown (Mills and Smith, 1962). Type III cells were unable to synthesize UDPGlUA and the polysaccharide as they lacked the enzyme UDPG dehydrogenase. The deficiency was not repaired by recombination and no Type III cells were produced. However a few binary capsulated cells of Type I -III were formed. This involved a gain by the recipient cells of the ability to synthesize at least the enzymes UDPGal - 4 - epimerase, UDPG dehydrogenase, UDPGlUA - 4 - epimerase, and GDPfucose synthetase, plus transferases and polymerase (s). A close linkage of these genes in Type I cells is thus indicated.

Study of the M or mucus antigen (Kauffmann, 1935; 1936) has shown the presence of a regulator gene controlling polysaccharide synthesis in this system (Markovitz, 1964). The antigen is not type specific and is thought to be produced by all Enterobacteriaceae under appropriate conditions of high salt concentration and low temperature (Anderson and Rogers, 1963). It has been called colanic acid (Goebel, 1963) and may be synthesized in addition to the normal extracellular polysaccharide. It consists of glucose, galactose, glucuronic acid and fucose. Its production leads to a further complication in control mechanisms. The normal feedback inhibition of pyrophosphorylases may have to be overcome to permit synthesis of both polymers.

A regulator gene R_I (cap R) closely linked to the "pro" locus on the chromosome was found to control the synthesis of colanic acid. As partial heterozygotes obtained by transduction or episomal infection were non-mucoid, the non-mucoid state is dominant. The wild type, cap R^+, is non-mucoid producing a regulator substance, while cap R^- produces none or a defective substance. Cap R controlled the activities of several enzymes involved in synthesis of the sugar nucleotide precursors of the polysaccharide. Mucoid strains showed higher levels of UDPGal - 4 - epimerase and GDPfucose synthetase than non-mucoid while levels of Glc - 6 - P dehydrogenase and phosphoglucose isomerase were similar. The structural gene for UDPGal - 4 - epimerase is in the "gal" region not adjacent to cap R, yet cap R regulated its activity both from a chromosomal and episomal position, (Markovitz, 1964). It did not regulate Glc-6-P dehydrogenase or phosphoglucose isomerase activities. The gene product of the cap R locus was thought to be protein (Markovitz and Baker, 1967). P-fluorophenylalanine, which may inactivate protein repressors, was found to convert a non-mucoid strain of E.coli to mucoid and the effect may be reversed by growth without pFA (Kang and Markovitz, 1966). The levels of the enzymes phosphomannose isomerase, UDPGal - 4 - epimerase and GDPfucose synthetase were elevated. It was concluded that derepression of capsular polysaccharide by pFA was due to its effect on the repressor produced by cap R (Kang and Markovitz, 1967[a]). Induction of colanic acid by pFA has also been shown in several Salmonella strains previously thought to be non-mucoid (Grant, 1968). Another regulator gene R_2 (cap S) was found to be involved in polysaccharide production. Mutation at this locus, which allows synthesis of colanic acid, did not elevate the levels of any of the enzymes. Thus

polysaccharide synthesis is not necessarily equated with derepression of the enzymes (Markovitz, Sydiskio and Lieberman, 1967). It was suggested that one of the enzymes may be altered so that it is not subject to feedback control (Markovitz, Lieberman and Rosenbaum, 1967).

The ability to produce a capsule may be carried episomally in a few instances. Thus the determinant for the K88 antigen of E.coli was transferable on an episome (Ørskov and Ørskov, 1966) but this may be a unique case as the K88 polymer is protein in nature (Stirm, Ørskov, Ørskov and Mansa, 1967). Another strain of E.coli carried four transfer factors, one a surface antigen giving rise to mucoid colonies (Hardy and Nell, 1967). The mucoid material may be colanic acid (Grant, 1968). A small part of the genetic material coding for some enzymes involved in its synthesis or a regulator gene may be transferred.

INTRODUCTION

SECTION C

MUREIN

Work on the composition of isolated, purified cell wall preparations led to the recognition of a "basal unit" (Work, 1957) common to both Gram-positive and Gram-negative bacteria. It contained the hexosamines, glucosamine and muramic acid together with D-glutamic acid, D- and L-alanine, L-lysine and/or m-diaminopimelic acid. In addition there may be other amino acids or sugars present. Various names have been given to it such as mucopeptide (Mandelstam and Rogers, 1959), peptidopolysaccharide (Sharon, 1963), and, most recently, murein (Weidel and Pelzer, 1964; Martin, 1966). It forms a highly polymerized cross-linked structure thought to be responsible for the mechanical rigidity of the cell wall.

Attention has been focussed on the murein component, its structure and synthesis, since it was discovered that some antibioties owe their selective toxicity to interference at some point in the synthesis of its precursors and their polymerization. The intermediates in its synthesis were characterized initially almost entirely due to the effects of these antibioties.

STRUCTURE

Parts of the structure of murein may be inferred by characterization of the intermediates which accumulate in cells treated with various inhibitors of cell wall synthesis. Penicillin has been of particular value in this respect, its point of inhibition being just before the formation of the final polymer. The products of partial acid hydrolysis (Perkins and Rogers, 1958) and bacteriolytic enzymes have provided the other major methods of studying the structure of murein.

Muramic acid was isolated in 1956 (Strange and Dark) from the spore coats of some Bacillus species. It was found to be a component of the cell walls of all bacteria and may be unique to procaryotic cells. It was realized that it might have an important role in the murein complex as it had a carboxyl group available for peptide bond formation in addition to a reducing group suitable for glycosidic bonds.

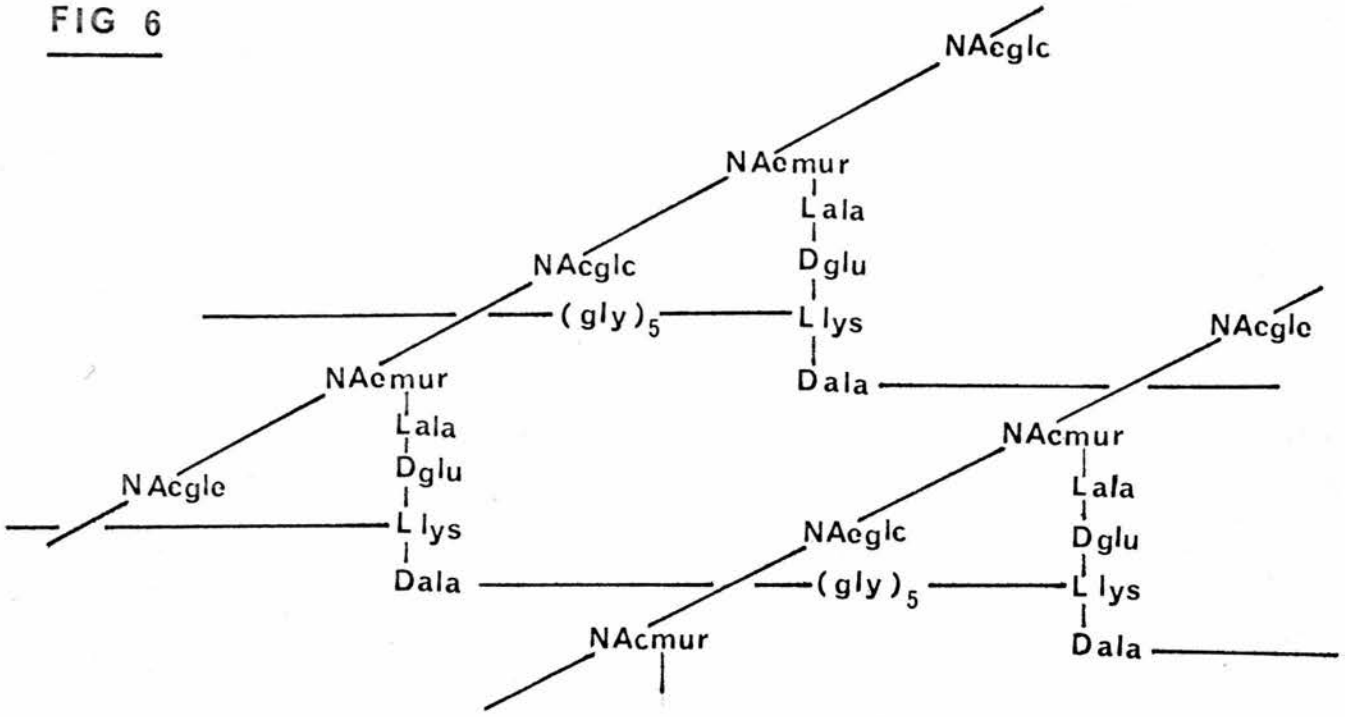
A group of sugar nucleotides was isolated from penicillin-treated cells of S. aureus by Park and Johnston (1949). They were identified as UDPNacetylmuramic acid and UDPNacetylmuramic acid peptides (Park, 1952). The largest nucleotide complex had the structure :

N-acetylmuramic
|
L ala - D glu - L lys - D ala - D ala (Strominger 1959)

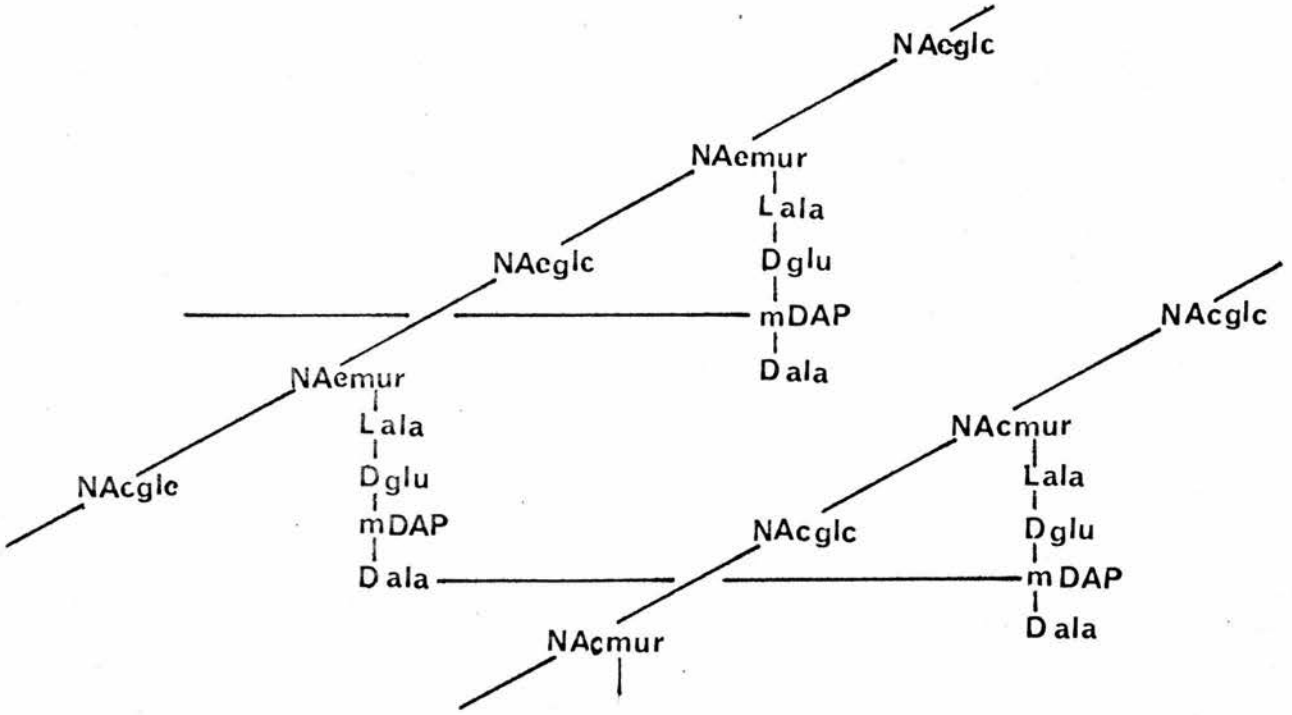
An equivalent nucleotide complex was found in penicillin-treated cells of E.coli but with m-diaminopimelic acid replacing L-lysine (Strominger, Scott and Threnn, 1959). A link was therefore established between the muramic acid residue and a pentapeptide.

One of the best known lytic enzymes is egg-white lysozyme shown by Fleming in 1922 to lyse cultures of Micrococcus lysodeiktieus and later found to attack cell walls of other Gram-positive organisms. There was release of only small amounts of reducing sugars and hexosamines (Salton, 1958). The simplest product was a disaccharide, N-acetylglucosamine - N-acetylmuramic acid which was probably linked β 1$\rightarrow$6 (Salton, 1956). Small amounts of a tetrasaccharide also occurred, possibly a dimer of this disaccharide joined β 1$\rightarrow$4. There may be a backbone of NAcglucosamine - NAcmuramic acid residues joined by alternate β1$\rightarrow$4 and β1$\rightarrow$6 linkages, the former

FIG 6



Structure of Murein of S. aureus (Tipper and Strominger, 1968)



Structure of Murein of E. coli

being lysozyme sensitive (Brumfitt, Wardlaw and Park, 1958). The susceptibility of a particular cell wall to digestion by lysozyme cannot be predicted from its chemical structure although O-acetyl groups may be important in resistance (Brumfitt, 1959).

In addition to the amino sugar-peptide component the murein may contain other amino acids, such as glycine in S. aureus (Mandelstam and Strominger, 1961). There are few free amino groups present in cell walls and cross links between peptide chains as well as polymerization of the amino sugar backbone may account for this (Salton, 1961). Furthermore there is release of only small amounts of low molecular weight compounds on treatment with lysozyme in the case of Gram-positive cells. Gram-negative organisms have been of great value in determination of structures as although they contain only a small proportion of murein, it is, however, not so cross-linked and is more susceptible to the action of lysozyme and other lytic organisms (Weidel and Primosigh, 1958; Pelzer, 1962).

Murein therefore consists of a backbone of alternate N-acetylglucosamine and N-acetylmuramic acid residues, most of which are substituted by peptide. Not all linkages between amino sugars are the same and not all peptide chains are identical. The degree of cross linkage tends to be higher in Gram-positive organisms and to involve other peptides; in Gram-negative organisms it may be replaced by a direct bridge between two amino acid residues in adjacent peptide chains (Pelzer, 1963).

Fig.6 illustrates the structures of the murein of S. aureus, a Gram-positive organism, and E.coli, a Gram-negative organism.

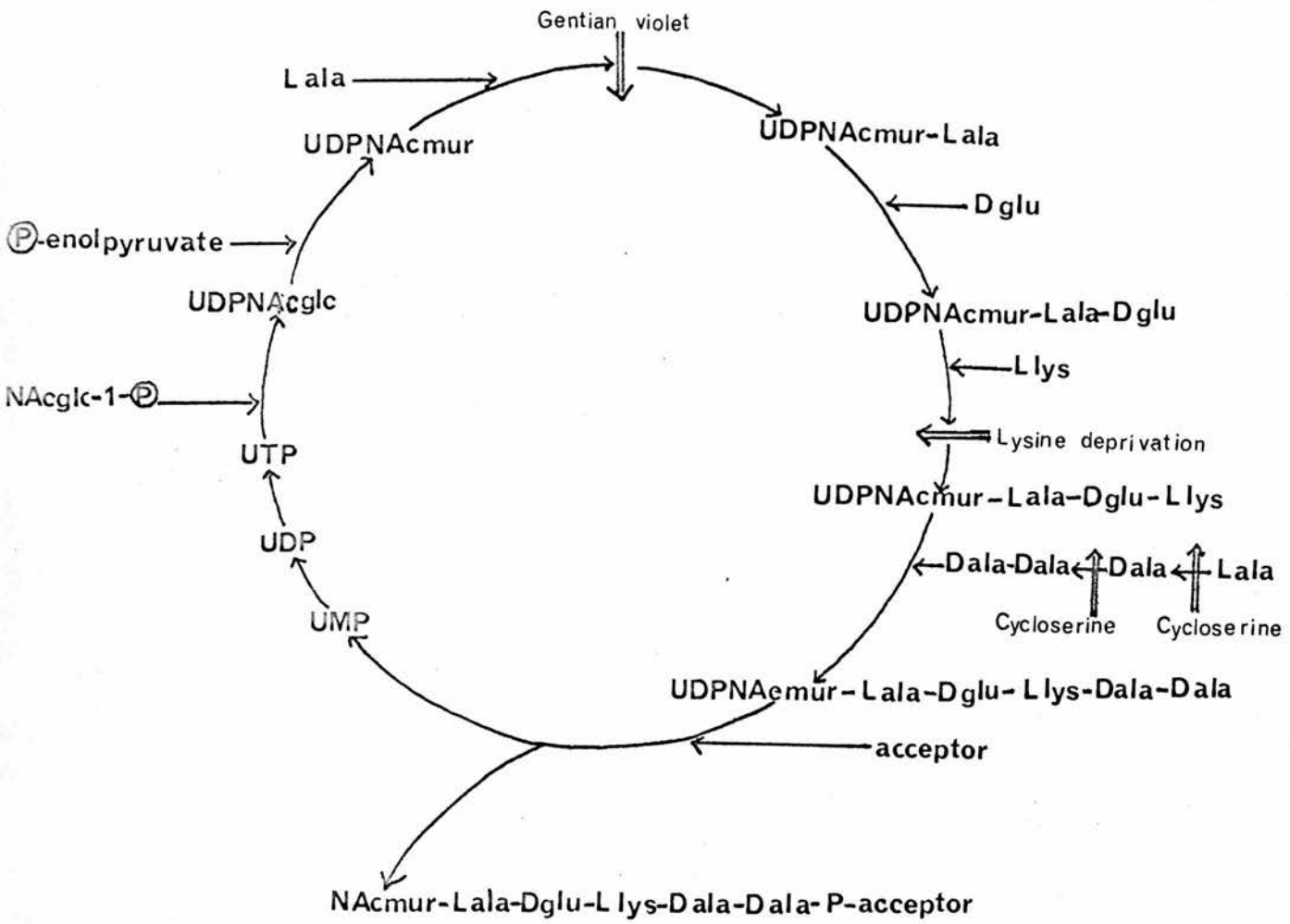


FIG 7 Synthesis of Nucleotide Precursors of Murein

(Strominger, 1962)

SYNTHESIS

Although the nucleotide UDPN-acetylmuramic acid pentapeptide of S. aureus had been identified in penicillin-treated cells in 1952 (Park), it was not until several years later that a connection with cell wall composition was suggested (Park and Strominger, 1957). A scheme was proposed whereby UDP acts as a carrier for a cycle during which UDPN-acetylmuramic acid pentapeptide is synthesized by stepwise addition of amino acids. This is summarized in Fig.7. The points of action of various inhibitors which cause accumulation of the intermediates of the cycle are also indicated. Each step leading to formation of UDPN-acetylmuramic acid pentapeptide has since been demonstrated in extracts of S. aureus, the enzymes purified and the products characterized (Ito and Strominger, 1960; 1962). ATP is required for every reaction and there is no prior activation of amino acids.

Until recently there was no direct evidence that the UDPN-acetylmuramic acid pentapeptide complex was used directly in the synthesis of murein. However it seemed probable if the similarities in structure of the complete murein and the nucleotides accumulated in the presence of various antibiotics were taken into account (Strominger, Park and Thompson, 1959). The mechanism of the incorporation of glucosamine into murein was not known. Transglycosidation from the uridine derivatives to an acceptor seemed an attractive theory, especially as it had been demonstrated for hetero- and homo-polysaccharides. Two points remained to be explained if that was the mechanism of synthesis. The first was the high glycine content of the cell walls of S. aureus and the inability to find, in most instances, a sugar - glycine complex. In the second place there was a drop in the ratio of alanine: glutamine from 3:1 in the nucleotide to 2:1 in the cell wall murein (Mandelstam and Strominger, 1961).

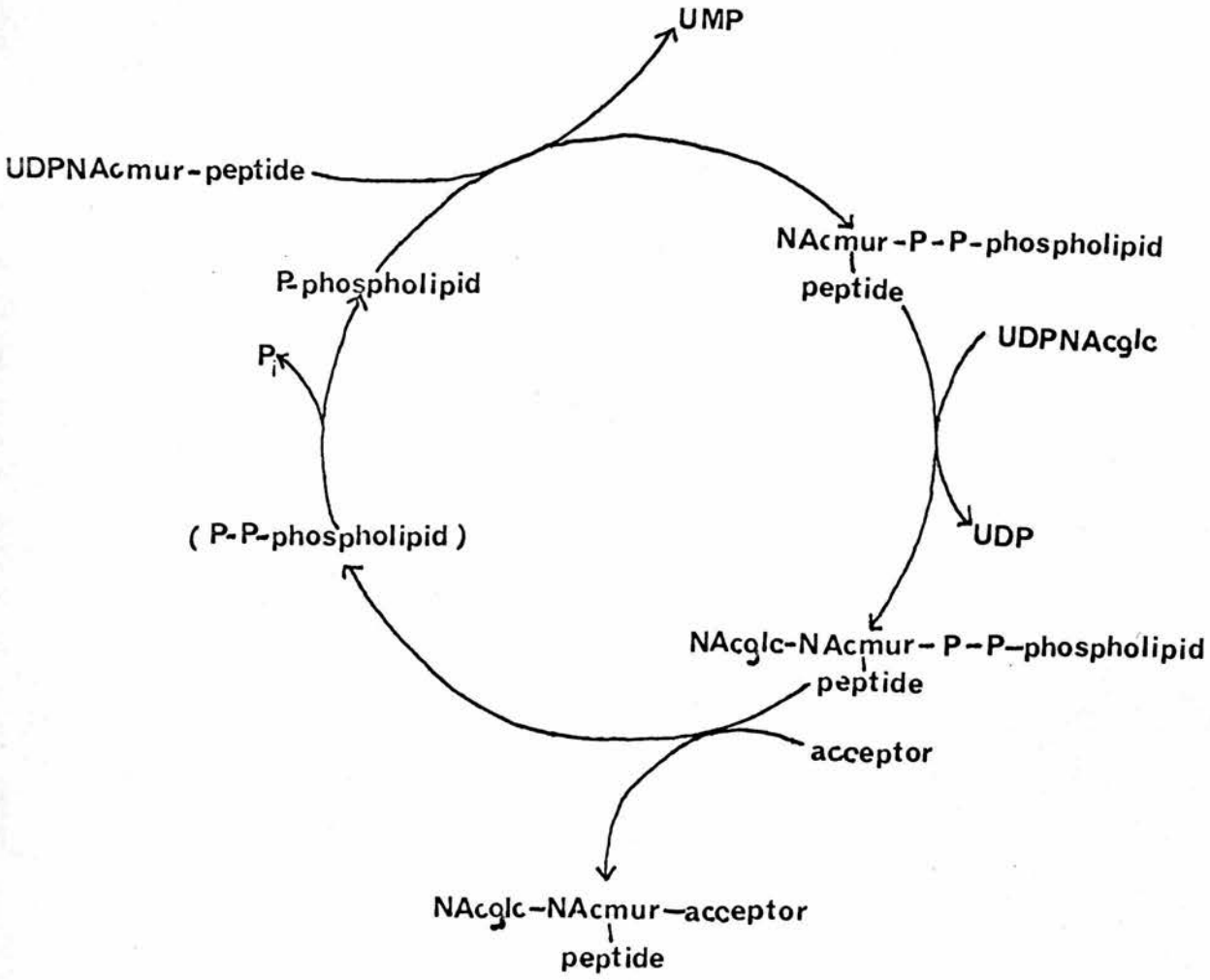


FIG 8 Synthesis of Peptidoglycan Units

(Anderson, Matsuhashi, Haskin and Strominger, 1967)

In 1964 Chatterjee and Park, using a particulate enzyme fraction from S. aureus demonstrated incorporation of UDPN-acetylmuramic acid pentapeptide into murein-like material in the presence of UDPN-acetylglucosamine. The product was lysozyme-sensitive and was not cross linked (Meadow, Anderson and Strominger, 1964). Incorporation of glycine could also be obtained in the presence of the two uridine nucleotides. This was prevented by chloramphenicol. It was suggested that a chain of glycine residues may be made, requiring sRNA, which was then incorporated into newly-formed murein backbone in a single reaction.

It had been noticed that during incorporation of N-acetylmuramic acid pentapeptide from the nucleotide there was release of UMP (Meadow, Anderson and Strominger, 1964). This led to the idea of an intermediate acceptor molecule and a year later the participation of lipid-linked intermediates in the synthesis of murein was demonstrated (Anderson, Matsuhashi, Haskin and Strominger, 1965). There was transfer of P - N-acetylmuramic acid pentapeptide to the lipid acceptor, followed by N-acetylglucosamine from UDPNAcglc to form a disaccharide-lipid intermediate. This was transferred to an acceptor with release of inorganic phosphate and carrier lipid. The cycle is illustrated in Fig.8. The acceptor of the disaccharide-lipid intermediate is probably an incomplete peptido-glycan strand. The cycle illustrates a possible membrane transport mechanism, showing how nucleotide precursors may be synthesized internally and by attachment to a lipid acceptor transported through the membrane. The lipid intermediates were extracted and purified from cells of S. aureus and M. lysodeikticus. Together with the appropriate enzyme fractions, they were used to synthesize murein in the absence of uridine nucleotides (Anderson, Matsuhashi, Haskin and Strominger, 1967).

Vancomycin inhibited this utilization and thus blocked the synthesis of murein. Bacitracin, which also inhibits cell wall synthesis, induced in S. aureus accumulation of pyrophosphate-lipid in addition to UDPN-acetyl-muramic acid pentapeptide. It may inhibit dephosphorylation of lipid-pyrophosphate thereby stopping the cycle (Siewert and Strominger, 1967).

The lipid carrier was found to be a C_{55} isoprenoid alcohol (Higashi, Strominger and Sweeley, 1967). It is similar in structure to the carriers used in synthesis of the O-antigenic polysaccharide in Salmonella and of mannan in M. lysodeikticus. The lipid intermediate may have to be modified before incorporation into the cell wall as, for example, in S. aureus by addition of NH_3 to the α-carboxyl group of glutamic acid and by addition of polyglycine chains. More complex forms of lipid intermediates may therefore be found.

The final stage in murein synthesis after formation of the linear peptidoglycan strands is cross linkage to complete the two- and three- dimensional structure. It was known that growth of S. aureus in the presence of penicillin caused increased incorporation of alanine and increase in number of N-terminal glycine residues. It was therefore suggested that cross linkage normally occurred by use of an interpeptide bridge composed of glycine residues. The terminal alanine of the pentapeptide would be released, this being sensitive to penicillin (Wise and Park, 1965). Peptidoglycan units which contained both D-alanine residues and unlinked polyglycine chains were later found to accumulate in cells of S. aureus treated with low concentrations of penicillin or cephalosporin (Tipper and Strominger, 1968). Penicillin is thought to act as a substrate analogue of Dalanyl-alanine reacting with the transpeptidase and inactivating it by acylation. It is believed that in normal synthesis of

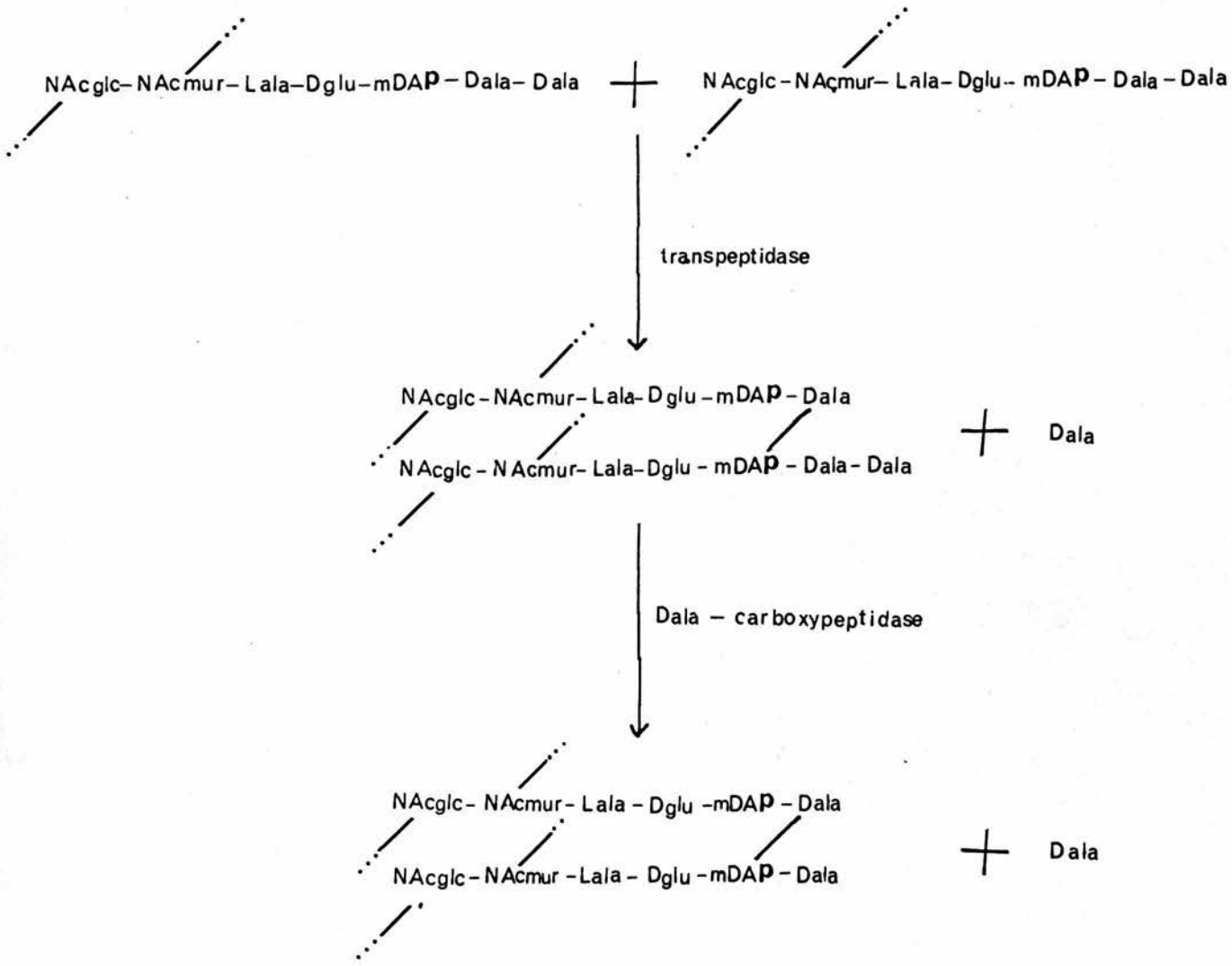


FIG 9 Cross–linkage of Peptidoglycan Units in E. coli

(Strominger, Izaki, Matsuhashi and Tipper, 1967)

murein, units may be added to the peptidoglycan with immediate cross linkage to another unit of a neighbouring chain. This is catalysed by a membrane bound transpeptidase utilizing the energy derived from a preformed peptide bond. Murein consisting of uncross-linked chains forms a weakened wall and the cell is susceptible to lysis.

Other Gram-positive organisms possess interpeptide bridges containing different amino acids such as threonine in Micrococcus roseus. Threonyl-sRNA participates in formation of these just as glycine-sRNA is used in S. aureus (Roberts, Strominger and Soll, 1968). Apart from the synthesis of such bridges sRNA is only known to be used in protein synthesis. In E.coli there are no interpeptide bridges. The end of one tetrapeptide is directly joined to the dibasic amino acid, m-diaminopimelic acid, in the next unit. The structure is illustrated in Fig.9. No long chain cross links are formed. These reactions catalysed by the enzymes transpeptidase and D-ala carboxypeptidase are sensitive to penicillin (Izaki, Matsuhashi and Strominger, 1966). In whole cells penicillin is not lethal, which may be due to a permeability barrier preventing its access to sensitive enzymes within the cell or to the presence of penicillinase enzymes.

Thus although murein is present outside the cell membrane, its precursors are synthesized inside the membrane, transported as lipid-linked units through the membrane, and finally polymerized and cross linked by transpeptidation outside.

Little information is available about the site(s) of cell wall synthesis in a growing cell. Use has been made of fluorescent-labelled antibody to the cell wall antigens, as in Streptococcus pyogenes (Cole and Hahne, 1962).

Growth does not occur diffusely by intercalation with the old wall as had been thought, but is initiated at at least two sites. A cross wall to complete the division is formed, and peripheral growth is initiated for the next division and cross wall. It has been suggested that there may be a moving site of active synthesis and once this has passed no more synthesis would occur at that point (Cole, 1965). The mesosome has been suggested as this moving active site. Polymerizing enzyme may similarily be at the site for a limited period only (Chatterjee and Park, 1964). Whether this suggestion applies only to the murein component or to all the polymers of the cell wall and whether their synthesis occurs simultaneously is not known at this time.

INTRODUCTION

SECTION D

LIPOPOLYSACCHARIDES

INTRODUCTION

For many years it has been recognised that the lipopolysaccharide component of the cell wall of Gram-negative bacteria contained the specific surface or O antigens utilized in serological classification, and was responsible for the endotoxic properties of the bacteria. Mutation from "smooth" (S) to "rough" (R) form caused loss of the O-antigenic specificity and virulence (Arkwright, 1920; 1921).

The structure of the lipopolysaccharide has been established by a combination of chemical, immunochemical, enzymic and genetical techniques. Information about its biosynthesis has been obtained concurrently with knowledge of its structure. These subjects have been extensively reviewed recently (Osborn *et al* 1964; Lüderitz, Staub and Westphal, 1966; Lüderitz, Jann and Wheat, 1968: Nikaido, 1968).

The lipopolysaccharide is thought to envelop the murein layer of the cell wall together with the phospholipid and protein components. No evidence has been obtained about its bonding to murein and protein, but it may be linked by physical bonds or through metallic ions. It consists of two lipid components, A and B, and a complex heteropolysaccharide containing from five to eight different monosaccharides. Sugar units always found in both S and R forms of *E.coli* and *Salmonella* include N-acetylglucosamine, KDO, heptose, glucose and galactose. In the former, however, various additional sugars are also normally found. It was proposed that the polysaccharide of S strains consisted of two

regions, the first containing the O-antigenic sugars, and the second, the R core, consisting of these five sugars linked to lipid A (Lüderitz, Kauffmann, Stierlin and Westphal, 1960). This concept has been shown to be essentially correct although it is now known that not every R mutant contains all five sugars of the core.

In Salmonella and E.coli strains, mutation to R form is therefore due to lack of synthesis or incorporation of the O-specific side chains. Mutation to the R form in Shigella flexneri is due to the same causes (Johnston, Johnston and Simmons, 1967). However the basal structure is not identical to that of Salmonella or E.coli although it contains the same sugars. It does not cross-react serologically with R anti-sera of either group (Johnston, Johnston and Simmons, 1968). In K. aerogenes and Aerobacter cloacae no similar chemotypes are found and a "basal structure" like that of Salmonella, Shigella or E.coli is not present (Sutherland and Wilkinson, 1966).

STRUCTURE AND SYNTHESIS OF THE R CORE

Information about the synthesis and structure of the core polysaccharide was obtained in the first place by use of R mutants blocked at some stage in synthesis of the core. A galactose-deficient mutant of Salmonella which lacked the enzyme UDPGal - 4 -epimerase was isolated. The lipopolysaccharide from these cells grown in the absence of galactose, lacked galactose and N-acetyl-glucosamine in addition to the O-specific sugars. Cell-free preparations of the mutant incorporated galactose from UDPGal into the deficient lipopoly-saccharide (Nikaido, 1962; Fukasawa and Nikaido, 1961). Another R mutant lacked the ability to form UDPG due to a mutation in phosphoglucose isomerase or UDPG pyrophosphorylase enzymes. Its lipopolysaccharide contained only the sugars

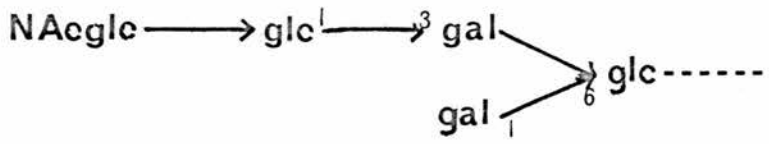


FIG 10. CORE STRUCTURE OF SALMONELLA LIPOPOLYSACCHARIDE

(Sutherland , Lüderitz and Westphal , 1965)

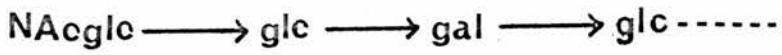


FIG 11. CORE STRUCTURE OF E. COLI LIPOPOLYSACCHARIDE

(Edstrom and Heath , 1967)

KDO and heptose (Fraenkel, Osborn, Horecker and Smith, 1963). Cell-free extracts were used to catalyse transfer of glucose from UDPG into the incomplete lipopolysaccharide (Rothfield, Osborn and Horecker, 1964).

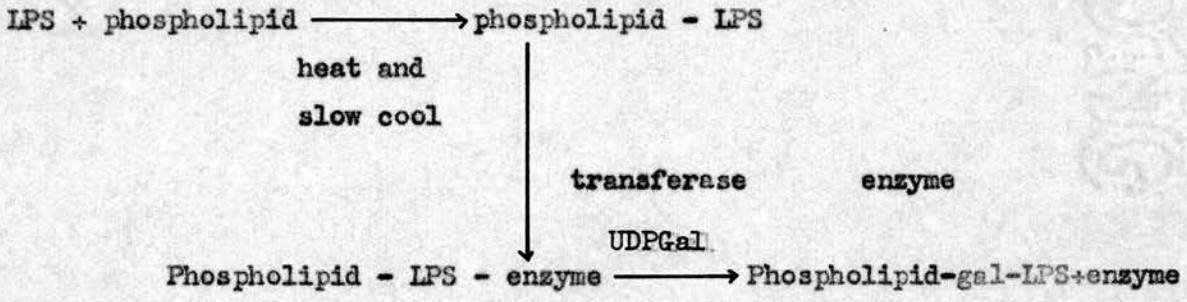
The composition of these mutants suggested that KDO and heptose were linked centrally to lipid A and that the R core was completed by addition of glucose, galactose and N-acetylglucosamine, these requiring to be present before the O-side chain could be added. After incorporation of galactose from UDPGal in the UDPGal - 4 - epimeraseless mutant, glucose was transferred from UDPG. In a similar manner N-acetylglucosamine was transferred from UDPNAcglc on to this glucose molecule (Osborn et al, 1964). The sugars of the R core thus appeared to be added sequentially in the order galactose, glucose and N-acetylglucosamine.

In addition to the results obtained from these biosynthetic reactions, the structure of the lipopolysaccharide was examined by chemical methods, such as by partial acid hydrolysis of the various incomplete lipopolysaccharides. This approach led to the same conclusion as had been envisaged from the order of sugar additions to the core, apart from the presence of a second galactose residue in the core (Sutherland, Lüderitz and Westphal, 1965). The core structure of Salmonella is shown in Fig.10.

Using a mutant of E.coli which was deficient in UDPGal - 4 - epimerase, cell-free preparations catalysed the sequential addition of galactose from UDPGal, glucose from UDPG, and N-acetylglucosamine from UDPNAcglc into incomplete lipopolysaccharide. The order of the monosaccharides constituting the core structure is thus the same as Salmonella species. However the core of E.coli does not contain a second galactose residue linked $\alpha 1 \longrightarrow 6$ with glucose as was found in Salmonella (Edstrom and Heath, 1964; 1967). The structure of

the E.coli lipopolysaccharide is shown in Fig. 11.

The cell-free extracts used for the incorporation experiments above contained both transferases and acceptors. Some of the transferase activities were present in the soluble fraction (Rothfield, Osborn and Horecker, 1964) and their specificities could be ascertained. Boiled cell wall-membrane fraction acted as acceptor in transferase reactions, whereas purified lipopolysaccharide was inactive. It was discovered that the lipid component, lipid B, was required in addition to purified lipopolysaccharide. If these were heated and slow-cooled together, they acted as acceptors. Phosphatidyl ethanolamine is the active component of lipid B. The transfer of a glycosyl unit occurred as follows, say galactose using lipopolysaccharide from cells deficient in UDPGal-4-epimerase :



(Rothfield and Takeshita, 1965; 1966).

The interaction of lipopolysaccharide and phospholipid was examined in the electron microscope. The lipopolysaccharide was inserted at intervals in the bimolecular leaflet structure of phospholipid. This process may occur during heating and slow-cooling in vitro. (Rothfield, Takeshita, Pearlman and Horne, 1966; Rothfield and Horne, 1967).

KDO is most probably the link between the core polysaccharide and lipid A (Osborn, 1963). The structure and biosynthesis of lipid A has yet to be studied in detail but it consists of subunits of N - β - hydroxymyristylglucosamine phosphate substituted by fatty acids and possibly acetyl at all remaining

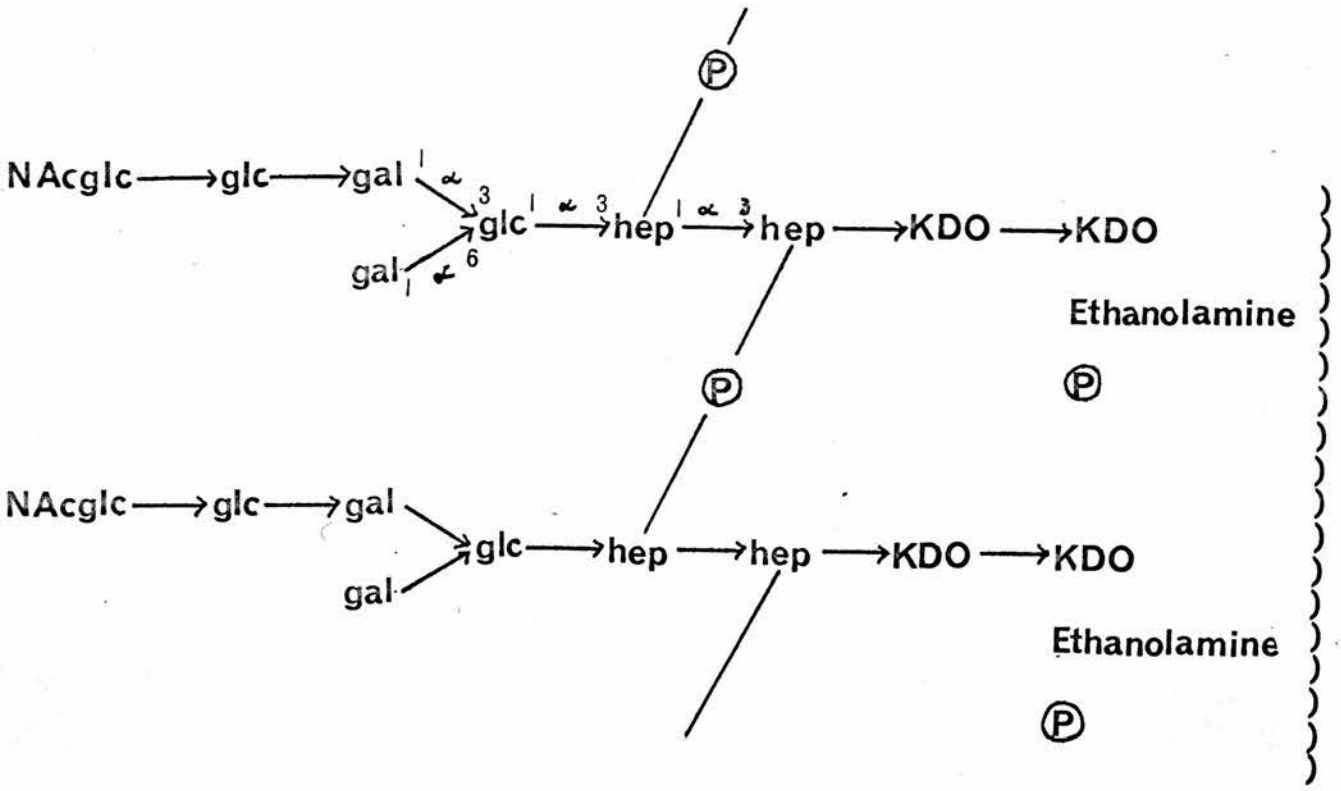


FIG 12 Core Structure of Salmonella LPS

(Dröge , Ruschmann, Lüderitz and Westphal, 1968)

hydroxyl groups (Westphal, 1960). N- β -hydroxymyristylglucosamine acted as acceptor of KDO from CMPKDO (Heath, Mayer, Edstrom and Beaudreau, 1966). The basal unit of the backbone of the polysaccharide consists of two residues of heptose, two phosphate, one ethanolamine and an indeterminant number of KDO. The structure of the unit was thought to be hep α 1→3 hep 1→[KDO] with phosphate and ethanolamine at unknown locations. Glucose was linked 1→3 to the first heptose (Cherniak and Osborn, 1966; Dröge, Lüderitz and Westphal, 1968). By comparing mutants with and without phosphate groups, cross links of phosphodiester bridges between adjacent heptose units were thought to occur (Dröge, Ruschmann, Lüderitz and Westphal, 1968). The proposed structure of the R core is shown in Fig. 12.

The structure of the core of E.coli lipopolysaccharide is now being determined similarily by use of R mutants (Wilkinson, Fuller, Lazen and Heath, 1968). The mutants were divided into two classes on the basis of their chemical composition. In the first class the cores were deficient in heptose, there were no phosphate cross linkages and free KDO or heptose - KDO oligosaccharides were liberated. In the second class the polysaccharides consisted of phosphorylated polymers containing 2 heptose residues and 1 KDO residue substituted by additional compounds - 1 glucose residue; 2 glucose residues and 1 galactose; 2 glucose, 1 galactose and 1 hexosamine; 4 glucose, 1 galactose and 2 hexosamine. The picture obtained from this information appears to be similar to that found in Salmonella species.

STRUCTURE AND SYNTHESIS OF O-SIDE CHAINS

In contrast to the R core, the O-side chains are formed by polymerization of repeating oligosaccharide units (Robbins and Uchida, 1963), containing from

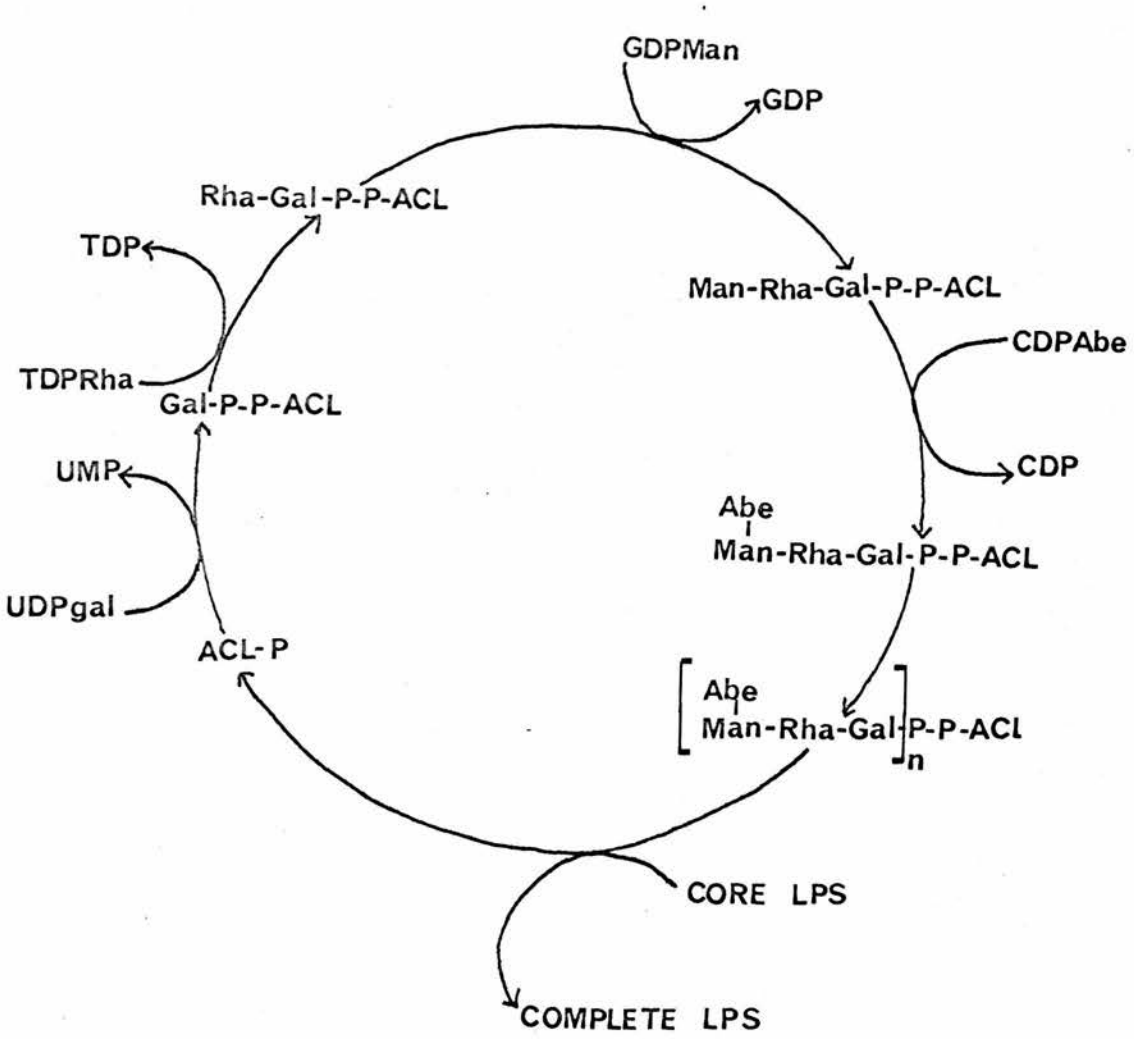
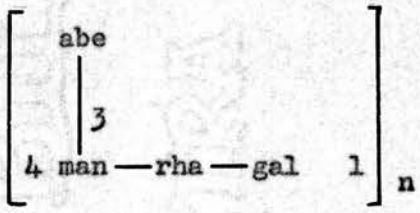


FIG 13

Lipid-linked Intermediates in S. typhimurium O-Antigen Biosynthesis

(Osborn and Weiner, 1967)

three to six sugars. It was suggested that these units may be synthesized as oligosaccharides before transfer to the growing chain. Such a carrier molecule on which the units may be assembled before transfer was found in 1965 (Wright, Dankert and Robbins; Weiner et al). Robbins' group used S.newington whose side chains contain mannose, rhamnose and galactose. The first reaction was transfer of gal-1 -Ⓟ from UDPGal to an intermediate carrier, a lipid called "antigen carrier lipid phosphate" (ACL - P). Rhamnose was then transferred from TDPRha to form a disaccharide intermediate followed by mannose from GDPMan. A similar scheme was discovered using a mutant of S. typhimurium lacking UDPGal - 4 - epimerase (Osborn and Weiner, 1967). The repeating unit in this case is a tetrasaccharide :



It was formed on the lipid carrier by sequential addition of the sugars from their nucleotide derivatives. This is illustrated in Fig. 13. Some evidence has been obtained for a similar oligosaccharide lipid intermediate in O-side chain synthesis in E.coli (Mayer, Edstrom, Beaudreau and Heath, 1966).

Following formation of the repeating unit on the lipid carrier, transfer to the core structure may occur by two possible methods. There may be polymerization of the repeating unit to polysaccharide -P-P-ACL and subsequent transfer to the R core, or sequential transfer of single repeating units may occur. In some R mutants lacking the ability to synthesize the core structure, there was accumulation of O-specific hapten groups as soluble polymers (Beckman, Subbaiah and Stocker, 1964). These were recovered from the aqueous

phase of phenol-extracted bacteria after the lipopolysaccharide component had been deposited by centrifugation. They reacted with O-antiserum. The O-hapten groups have also been identified in cell-free systems, such as in a mutant of S. typhimurium lacking UDPGal - 4 -epimerase (Osborn and Weiner, 1968), in addition to being formed in vivo in certain R strains. The average length of the hapten groups is 30 repeating units and they are firmly bound to the particulate cell envelope fraction (Kent and Osborn, 1968[a]). However, proof that polymerization of O-antigenic chains precedes their transfer to the core and that polysaccharide-P-P-ACL is an intermediate was not obtained until recently (Kent and Osborn, 1968[b]). Analysis of the kinetics of the uptake of labelled monosaccharides into O-hapten and lipopolysaccharide indicated the presence of an intermediate of high turnover rate. In addition pulse chase experiments showed rapid transfer of O-hapten from the lipid carrier to lipopolysaccharide. It was concluded that polymerization of repeating units occurs before transfer from ACL. But transfer of single units is not entirely excluded. It has been shown that a single incomplete repeating unit may be transferred to the core lipopolysaccharide from ACL (Nikaido, 1965) and that some SR mutants contain O side chains consisting of 1 repeating unit (Naide et al, 1965). The first repeating unit may be added to the core singly and the others as a polymer, and genetically two loci synthesizing different polymerases may be involved (Mäkelä, 1966).

The disaccharide lipid intermediate, rha-gal-lipid of S.newington mentioned above was prepared and purified in sufficient quantity to allow structural studies of the lipid and its linkage to the first sugar, galactose (Wright, Dankert, Fennessey and Robbins, 1967). On mild acid hydrolysis the free

disaccharide rha-gal, pyrophosphate and ACL were formed. Pyrophosphate was recognized by use of the enzyme pyrophosphatase. On mild alkaline hydrolysis disaccharide-phosphate and ACL-phosphate were formed. Infra-red analysis of the latter indicated a terpene structure. Mass spectrometric analysis of the ACL produced by mild acid hydrolysis suggested a C_{55} compound composed of 11 isoprene units. From these results and other data ACL was found to be a C_{55} polyisoprenyl alcohol linked to the first sugar by a pyrophosphate bond. In structure it is thus very similar to the carrier used in the synthesis of the murein of cell walls and the mannan of M. lysodeikticus.

The mechanism of chain elongation was found by pulse labelling experiments to be like that in synthesis of proteins and unlike that in synthesis of glycogen, starch and core polysaccharide. There was growth of the chain from the reducing end of the polymer. This means the repeat unit polymer is added to the non-reducing end of the lipid-linked monomer (Robbins, Bray, Dankert and Wright, 1967). It is illustrated below :

□-□-□-P-P-ACL + ■-P-P-ACL → □-□-□-■-P-P-ACL + P-P-ACL

□ = Polymer Unit

CONTROL OF SYNTHESIS

Genetical classification of R mutants has shown that two loci are concerned. The first, rfa, maps near "ilv-xyl" region and the second, rfb near "his" (Subbaiah and Stocker, 1964; Stocker, Wilkinson and Mäkelä, 1966).

rfa mutants are unable to synthesize a complete R core structure due either to a defect in structural enzymes or in transferases. Thus, although in some cases, the O-antigen hapten is synthesized, it cannot be added to the core. rfa mutants may be divided into different classes by recognition and mapping of the enzyme involved, chemical composition of the R core and

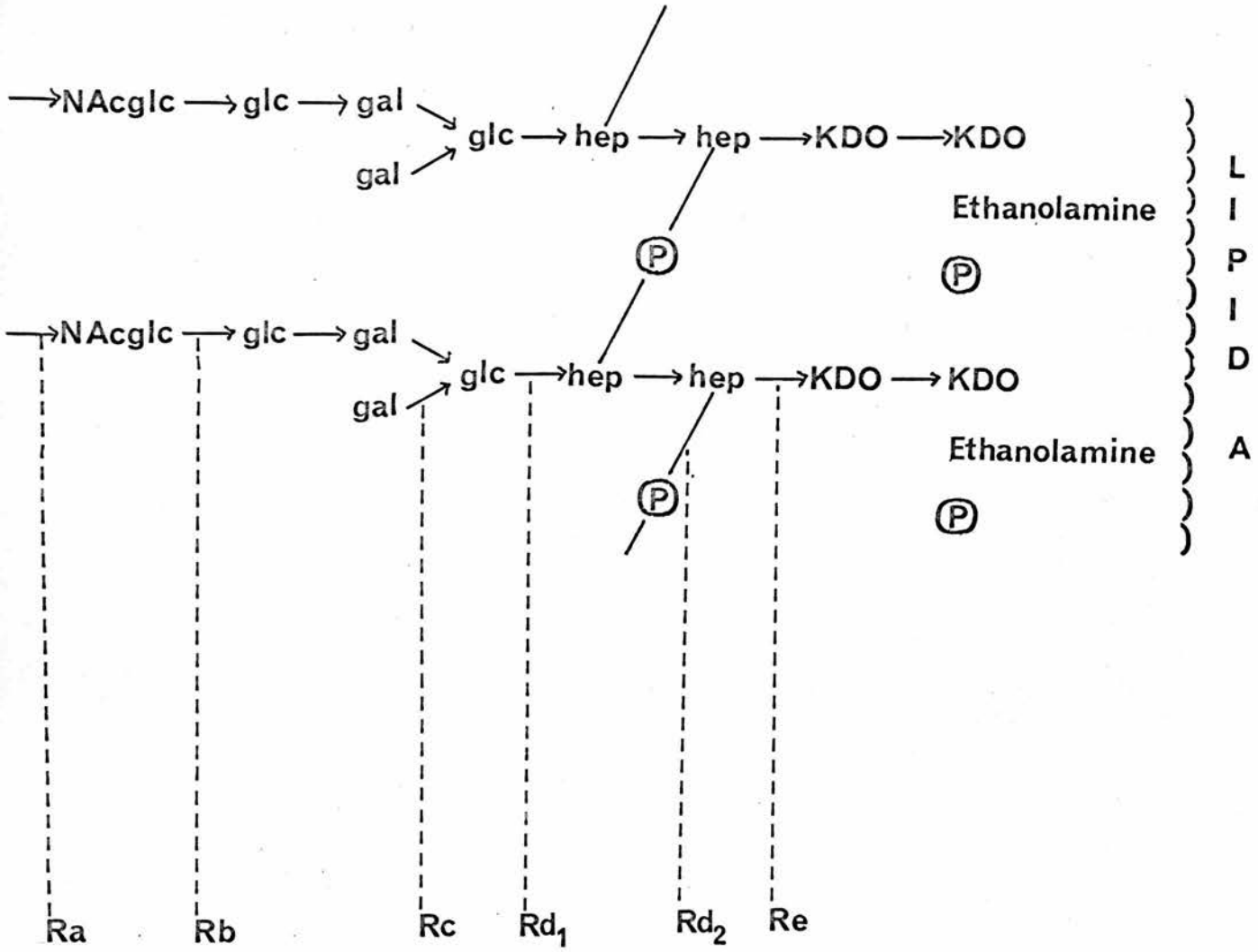


FIG 14

Core Structure of Salmonella LPS and R-Mutants

serological cross reactions. Six classes are now known which are shown in Fig.14. These provide a final proof of the sequential sequence of sugars in the core, which had been demonstrated previously by enzyme reactions.

rfb mutants have a complete R core but are unable to synthesize the O-side chains. In S. typhimurium this region has been mapped in considerable detail utilizing histidine-requiring mutants with deletions extending for varying lengths into it (Nikaido, Levinthal, Nikaido and Nakane, 1967). Genes determining synthesis of each nucleotide such as TDPRha were located together and in several instances more than one gene coded for one enzyme. The genes for transferases and polymerase were also thought to map in the rfb region. Mutation in these enzymes was much more common than mutation in enzymes involved in synthesis of the nucleotide sugars. One group of mutants, the SR mutants of groups B and D Salmonella, was deficient in a polymerase enzyme which did not map at rfb. It mapped at a locus called rfc between "trp" and "gal" (Mäkelä,1966). There was transfer of units from the lipid carrier without polymerization, so that the O-side chains consisted of 1 unit instead of about 30 repeating units (Naide et al, 1965).

Control of lipopolysaccharide production may be at the level of synthesis of the nucleotide sugar precursors or of the lipopolysaccharide itself. The former is controlled, at least in a number of instances, by feedback inhibition (Bernstein and Robbins, 1965; Kornfeld and Ginsburg, 1966) without alteration in the levels of enzymes. Efficiency of this process may vary as accumulation of nucleotides occurs in some strains. Hydrolytic enzymes may also affect nucleotide sugar levels in the cell (Glaser, Melo and Paul, 1967). The composition of the O-side chains may be affected by the presence of phage. The phage genome may code for new modifying enzymes and polymerases and, at the same time, produce repressors inhibiting the enzymes and depolymerases of the host,

(for examples see Uchida, Robbins and Luria, 1963; Fuller, Etievant and Staub, 1968).

Nothing definite is known about the control of the synthesis of lipopolysaccharide itself and its correlation with synthesis of other polymers of the cell wall. O-side chains are not synthesized in the presence of chloramphenicol which may indicate an overall mechanism of control (Robbins, Keller, Wright and Bernstein, 1965). However a lysine-requiring mutant of E.coli was found which secreted large amounts of lipopolysaccharide into the medium together with phospholipid and protein. This occurred in the absence of lysine and hence of cellular protein synthesis (Work, Knox and Vesk, 1966). In another instance, lipopolysaccharide was produced without simultaneous formation of murein in a L-form of Proteus (Martin, 1964). Thus the organization of the whole macromolecular structure of the cell wall and its relationship with the membrane still remains to be established.

INTRODUCTION

SECTION E

GLYCOGEN

The intracellular polysaccharide, glycogen, has glucose as its only constituent. It has been found widely amongst Gram-positive and Gram-negative genera. It is synthesized under conditions of nutrient inbalance, often where nitrogen is the limiting growth factor, and is thought to be used for cell maintenance in the absence of an exogenous source of carbon and energy.

STRUCTURE

Structural studies have been carried out by enzymic and chemical methods. The enzymic approach has included the use of β-amylase which splits maltose units from the outer chains of the glycogen molecule (French, 1960), α-amylase which catalyses hydrolytic cleavage of α-1, 4 linkages (Fisher and Stein, 1960), and glycogen phosphorylase which, in the presence of inorganic phosphate, removes glucose from the outer chains of the polysaccharide by splitting α-1,4 linkages (Walker and Whelan, 1960). The degree of branching has been determined using the phosphorylase enzyme in conjunction with a debranching enzyme which splits α-1,6 linkages (Illingworth, Cori and Larner, 1952). Chemical approaches have included periodate oxidation and methylation analysis (Manners, 1957; Kjolberg, Manners and Wright, 1963).

Bacterial glycogen has been shown to be similar in structure to animal glycogen, consisting of a highly branched glucose polymer in which chains of α-1, 4 residues have branch points of α-1, 6 residues. The ratio of linear to branch linkages is around 12 to 1 although there is considerable variation according to the source of glycogen (Manners, 1957).

SYNTHESIS

The work of Cori, Schmidt and Cori (1939) demonstrated transglycosidation from a phosphate ester of a sugar during synthesis of a polysaccharide. Liver phosphorylase was known to break down glycogen to glucose - 1 - phosphate in the presence of inorganic phosphate. The reverse reaction was shown to occur also, chains of glucose units being synthesized from glucose - 1 - phosphate. However an alternative synthetic pathway was suggested to account for the formation of glycogen under conditions where either the phosphorylase enzyme was deficient, or the equilibrium favoured the catabolic reaction. The enzyme glycogen synthetase (UDPG : α-1,4 - glucan - α -4 - glucosyl transferase) provided an alternative method of synthesis (Leloir and Cardini, 1957) and has been found in a number of bacterial strains. It was present in the 105,000g supernatant fraction of Arthrobacter (Greenberg and Preiss, 1965), but in the 105,000g particulate fraction of E. coli (Preiss and Greenberg, 1965). ADPG is by far the best glucosyl donor but the enzyme shows slight activity with UDPG and TDPG (Greenberg and Preiss, 1964). This is in contrast to the synthetases of animals and yeasts which utilize UDPG as the main glucosyl donor. Mutants lacking UDPG pyrophosphorylase are not altered in their ability to store glycogen (Sigal, Cattanéo and Segel, 1964); but mutants lacking the synthetase do not form any glycogen (Damotta, Cattanéo, Sigal and Puig, 1968).

The glycosyl donor ADPG is synthesized by the enzyme ADPG pyrophosphorylase from ATP and glucose - 1 - phosphate. The presence of this enzyme has been demonstrated in a variety of bacterial species including Agrobacterium tumefaciens, K. aerogenes, E.coli, M.lysodeikticus and Arthrobacter (Shen and Preiss, 1965). It was found in the 105,000g supernatant fraction.

The branch points of the glycogen structure are synthesized by transglycosidation catalysed by a branching enzyme (α - 1,4 - glucan : α - 1, 4 - glucan - 6 - glycosyl transferase). Splitting of an α- 1,4 - linkage occurs and a chain of 6 - 8 glucose residues is transferred to an internal glucose unit with formation of α- 1,6 linkage. The branching enzyme has been found in E.coli (Sigal, Cattanéo, Cambost and Favard, 1965) and Arthrobacter globiformis (Zevenhuizen, 1964) and is active with both amylose and amylopectin.

An additional glycosyl compound has been found in wild type E.coli and in mutant cells deficient in UDPG pyrophosphorylase (Mayer, Rapin and Kalckar, 1965). It is not degraded by the enzyme amylase, thus being unlike glycogen in structure. The main components are glucose and hexosamine and linkage appears to be 1$\longrightarrow$2 and/or 1$\longrightarrow$6. As has been established in the synthesis of glycogen, there is the possibility that a glycosyl donor other than UDPG is used. Alternatively the very small quantity of UDPG probably present in the nucleotide pool of the mutant lacking UDPG pyrophosphorylase may be sufficient to synthesize this polymer, although it is not enough to allow the synthesis of the core structure of the lipopolysaccharide.

Glycogen synthetase and branching enzyme were believed to act only on polymeric substrates. Primer requirements for the former have been demonstrated in cell-free systems. In E.coli and Arthrobacter, α - 1,4-polyglucans or some oligosaccharide of the maltodextrin series were shown to act as primers for transglucosylation from ADPG (Preiss and Greenberg, 1965). In K. aerogenes, amylopectin, glycogen or dextrins acted as primers, the structure formed being independent of the primer used (Kindt and Conrad, 1967).

In 1961, synthesis in the absence of a primer was shown to occur in muscle by the action of phosphorylase on glucose - 1 - phosphate (Illingworth, Brown and Cori, 1961). Early intermediates were 2 - 6 glucose units attached to the enzyme, followed by rapid chain elongation. De novo synthesis in bacterial systems has proved difficult to demonstrate due to the problem of ensuring complete absence of any possible primer molecules. In late log cells of K. aerogenes glycogen was present at a concentration of 1% that found in stationary phase cells. This material was in such small quantity that it would not act as primer for glycogen synthetase (Koeltzow, Epley and Conrad, 1968). If it was assumed that no primer was present in late log cells, de novo synthesis of glycogen from ADPG was demonstrated (Gahan and Conrad, 1968). There was several hundred-fold stimulation of synthesis by addition of the 176,000g supernatant fraction of the cells, glycogen, bovine serum albumin, Triton X-100 or lipopolysaccharide of K. aerogenes. These may act as activators of glycogen synthetase rather than as acceptors for glycosyl residues. If the enzyme preparation containing the glycogen synthetase was allowed to stand at 0° or -10°, it lost activity and was activated only by bovine serum albumin or glycogen, thus becoming more like a primer-requiring system.

During synthesis of glycogen, chains are normally extended by successive additions of glucose units from ADPG to the non-reducing ends of the primer, which then act as substrates for the branching enzyme. However during de novo synthesis of glycogen in K. aerogenes, there are successive additions of oligosaccharides to the reducing terminals of the growing molecule (Gahan and Conrad, 1968). This is similar to O-antigen synthesis in lipopolysaccharides except that no lipid carrier was shown to be involved.

CONTROL OF SYNTHESIS

Cellular regulation of glycogen synthesis and breakdown occurs as under certain growth conditions, often nitrogen limitation, glycogen accumulates. It is re-used in the absence of an exogenous source of carbon energy. Where growth is limited and there is excess of carbon, the cell may require DNA, RNA and proteins to a much smaller extent and ATP may be produced in excess. Other intermediates may also accumulate. ADPG pyrophosphorylase is activated by some of these intermediates such as fructose - 1,6 - di Ⓟ glucose - 1,6 - di Ⓟ glyceraldehyde - 3 - Ⓟ Ⓟ -enol pyruvate. It is inhibited by phosphate, AMP and ADP so that where ATP is being utilized faster than its synthesis, these will reverse the process. Regulation of glycogen synthesis may thus be at the level of ADPG pyrophosphorylase (Preiss, Shen, Greenberg and Gentner, 1966). In animals and yeasts, on the other hand, where UDPG is the glycosyl donor, control may be at the level of glycogen synthetase which is stimulated by glycolytic intermediates like glucose - 6 - Ⓟ (Rothman and Cabib, 1966).

Mutants have been found lately altered in their ability to store glycogen (Damotta, Cattanéo, Sigal and Puig, 1968). There may be lack of the enzyme glycogen synthetase in which case no polysaccharide is formed. Branching enzyme may be deficient, so that a linear polyglucose molecule with no branch points is produced. In a third mutant, glycogen accumulates in amounts exceeding 40% of the cell dry weight. There is no de-repression of the three enzymes involved in synthesis of glycogen from glucose - 1 - Ⓟ Some normal inhibitor of synthesis may be removed. This mutation, the mutation in glycogen synthetase and in branching enzyme may be co-transduced which indicates close linkage of these genes on the chromosome (Sigal and Puig, 1968).

The present work has been concerned with the production of extracellular polysaccharide by two capsulate strains of K. aerogenes. The conditions necessary for this production both with regard to the environment and the state of the culture have been examined. The specific activities of enzymes concerned in the synthesis of the nucleotide sugars, precursors of the sugar units in the polysaccharide, have been measured under varying conditions. Some mutants unable to synthesize the polysaccharide under any circumstance, and others with temperature-dependent synthesis have been found and their characteristics determined. Attempts have been made to obtain a cell-free system of strain A4 which will synthesize polysaccharide material from the nucleotide sugar precursors.

MATERIALS AND METHODS

BACTERIAL STRAINS

K. aerogenes Type 54 Strain A1. Described by Wilkinson, Duguid and Edmunds (1954) and Dudman and Wilkinson (1956).

K. aerogenes Type 8 Strain A4. Described by Dudman and Wilkinson (1956).

They were cultured routinely on nutrient agar at 35° and maintained on nutrient agar slopes in screw - capped vials.

BACTERIOPHAGE STRAINS

Strains were provided by Dr. I.W. Sutherland, Department of General Microbiology, University of Edinburgh, using techniques described previously (Sutherland, 1967) except bacteria were removed by membrane filtration not chloroform.

Strain F_{40} - isolated on *K. aerogenes* A1

Strain F_{41} - isolated on *K. aerogenes* A4

CHEMICALS AND COMMERCIAL ENZYME PREPARATIONS

The following compounds were obtained commercially as the free form of the sodium or barium salt; barium salts were converted to sodium by treatment with 10% (w/v) $NaSO_4$:

ADP, ATP, ADPG, UDP, UTP, UDPGal, UDPG, UDPGlUA, GDPM, NAD, NADH, NAPD, NADPH, Glc-1-P, Glc-6-P, Fru-6-P, Man-6-P, glc, gal, fuc, man, rha, glUA, NAcglc, $NaPP_i$

N-Methyl-N-Nitroguanidine (MNG) was obtained from Koch-Light, Colnbrook, Bucks; acriflavine from B.D.H. Ltd., Poole, Dorset; ethyl methane sulphonate (EMS) from Kodak Ltd., Kirby, Liverpool; penicillin as Crystapen, benzyl

penicillin, from Glaxo Ltd., Greenford, Middlesex; chloramphenicol from Park Davis & Co., Hounslow, London.

The following isotopes were obtained commercially from the Radiochemical Centre, Amersham.

^{14}C-glc 3.77 mC/mM; ^{14}C-UDPG 76 mC/mM; ^{14}C-UDPGal 5.9mC/mM; ^{14}C-UDPGlUA 24.5 mC/mM; ^{14}C-Glc-6-Ⓟ 3.5 mC/mM Each was uniformly labelled.

The following enzymes were obtained commercially :

Glc-6-P dehydrogenase, hexokinase, phosphoglucomutase, phosphomannose isomerase, (all from Boehringer Corporation, London) and UDPG dehydrogenase (Sigma Biochemicals, London). Enzymes suspensions in $(NH_4)_2SO_4$ were used directly; freeze dried preparations were made up in distilled water before use. Lysozyme was obtained from Koch-Light, Colnbrook, Bucks

MEDIA

Sterilization was carried out by autoclaving at 15lb. per sq. in. (121°) for 15 minutes. Supplements were sterilized separately by autoclaving or by membrane filtration and added aseptically. Millipore filters (Millipore U.K. Ltd., Wembley, Middlesex) of pore size 0.45 μ or 0.22 μ were used for membrane filtration. Swinney hypodermic adaptors (Millipore Ltd.) were used for small volumes and Millipore pyrex filter units for larger volumes.

Nutrient broth and nutrient agar

Nutrient broth was prepared from Oxoid No. 2 nutrient broth powder (Oxoid Ltd., London) and nutrient agar by the addition of Oxoid No. 2 Ion Agar. In some experiments nutrient broth was supplemented by the addition of glucose sterilized separately to give a final concentration of 1%.

Yeast extract medium

This has been described by Sutherland and Wilkinson (1965). It contained g/liter medium : yeast extract (Oxoid), 1.0; casamino acids (Difco, technical), 1.0; Na_2HPO_4, 10; KH_2PO_4, 3; $MgSO_4 \cdot 7H_2O$, 0.2; K_2SO_4, 1.0; NaCl, 1.0; $(NH_4)_2SO_4$, 0.03; $CaCl_2$, 0.01; $FeSO_4$, 0.001. Glucose solution, 20% (w/v), was sterilized separately and subsequently added to the main solution after sterilization to give a final concentration of 2% (w/v). The medium may be solidified by the addition of 1.5% ion agar.

Minimal medium

The medium contained the same constituents as described for yeast extract medium but without yeast extract or casamino acids. It has been described by Wilkinson, Duguid and Edmunds (1954). It may be supplemented by sugars other than glucose to give a final concentration of 2%.

Minimal pFA medium

Minimal medium supplemented with D, L-p-fluorophenylalanine (pFA) was used. pFA was sterilized separated by membrane filtration and added to give a final concentration of 8×10^{-5} M in liquid media, and 5×10^{-6} M in solid media (Kang and Markovitz, 1966).

EMB Agar

EMB agar contained g/liter medium : Bactopeptone (Oxoid), 3.0 : K_2HPO_4, 0.6 ; cosin yellow, 0.4 ; methylene blue, 0.65 ; ion agar, 1.5. The constituents were dissolved by steaming and the pH adjusted to 7.0. 10% (w/v) glucose or other sugar solution was sterilized separately and added to the basic solution after sterilization to give a final concentration of 1% (w/v).

BUFFERS

All buffers were stored at 4°.

Saline

0.85% (W/v) NaCl in distilled water

Tris-HCl
Phosphate
Acetate

These were prepared of desired molarity according to the methods outlined in "Medical Microbiology" (Cruickshank, 1965).

Glycine - NaOH

A 1.0M solution of glycine was brought to the desired pH by the addition of 10N NaOH.

Cysteine - NaOH

A 0.1M solution of cysteine - HCl was brought to the desired pH by the addition of 5N NaOH. This buffer was unstable and was freshly prepared before use.

Triethyl Ammonium Acetate

A 1.0M solution of triethylamine in distilled water was adjusted to the pH required with glacial acetic acid.

CULTURAL METHODS

Liquid cultures were grown in Erlenmeyer flasks (capacity 250 ml, 500 ml, 1.5 l or 2.0 l) containing half the nominal volume and shaken at 2-300 rpm in

an orbital incubator (Gallenkamp, London). Shaking water baths were also used.

Production of extracellular polysaccharides

Yeast extract or minimal medium was used to give maximal polysaccharide production. Growth was carried out in sterile enamel trays containing 1 liter medium as described by Sutherland and Wilkinson (1965). The medium was inoculated from cultures grown in nutrient broth. Incubation was carried out for 3-4 days at 30°.

Production of lipopolysaccharides

Cells were grown overnight at 35° normally in nutrient broth.

Production of nucleotides

Nutrient broth was used as the growth medium to minimize extracellular polysaccharide production. When a large quantity of cells in the exponential phase of growth was required, a non-aseptic technique was used. 5 l overnight nutrient broth cultures were used to inoculate 30 l of 1/10th normal strength nutrient broth in a Hoover washing machine. The washing machine had been previously filled with distilled water, brought to the boil, covered and allowed to cool overnight. When the temperature had reached the required level, nutrient broth powder was added followed by the inoculum. The agitator on the machine was switched on and growth continued for 2-4 hours. When the cells were not required in such large quantities, Erlenmeyer flasks were used.

Growth of cells for enzyme assays

Cultures were normally grown in yeast extract medium or minimal medium

at 30° overnight.

Mutagenesis

1) Old broth culture

Nutrient broth cultures in test tubes were incubated at 35° for 10-12 days. Dilutions were made into sterile saline and 0.1ml aliquots spread over the surface of suitable media.

2) Acriflavine

Nutrient broth cultures containing acriflavine (0.1mg/ml) were incubated for 48 hours at 37°. Appropriate dilutions were spread over suitable media.

3) Ethyl methane sulphonate

EMS was used as described by Loveless and Howarth (1959) except that after treatment with the mutagen the cells were incubated in nutrient broth overnight before plating out.

4) Manganous chloride

The method of Holloway (1955) was used.

5) 2-aminopurine

Overnight broth cultures were diluted to 10^4 cells/ml in broth containing 2-aminopurine (200 µg/ml). This medium was freshly prepared and sterilized by membrane filtration just before use. The cultures were incubated for 48 hours at 35° and appropriate dilutions spread over plates of suitable media.

6) N-methyl-N-nitroguanidine (MNG)

The technique used was similar to that of 2-aminopurine except that MNG was present at a concentration of 100 µg/ml.

7) Caffeine

The method used was similar to that of 2-aminopurine except that caffeine

was present at a concentration of 0.5%.

8) Nitrite

Overnight broth cultures were washed and suspended in saline. 0.5mls washed suspension was added to 4.5mls 0.5M acetate buffer pH 4.4. 0.1ml M $NaNO_2$ solution was also added. This was freshly prepared and sterilized by membrane filtration just before use. After 15 minutes incubation at 37°, appropriate dilutions were spread over suitable media.

9) 45°

Broth cultures were incubated for 48 hours at 45°. Dilutions were spread over appropriate media.

10) Ultra-violet light

5 mls overnight broth cultures were washed, resuspended in saline and poured into sterile Petri dishes. They were irradiated for periods of 1-4 minutes at a distance of 30cms using a portable uv-ray lamp (Model II, Hanovia, Slough, England). Dilutions were plated out directly on to suitable media or incubated for 24 hours at 35° in nutrient broth before plating out.

11) γ-rays

Overnight broth cultures were washed, resuspended in saline and 3mls used to fill special sterile containers. These were irradiated for period of 1-3 minutes using a $Cobalt^{60}$ source. Appropriate dilutions were plated out directly on to suitable media or incubated for 24 hours at 35° in nutrient broth before plating out.

Penicillin Selection

Penicillin selection was carried out by one of two alternative methods.

1) The method described by Gorini and Kaufman (1960) was used. Penicillin treatment was carried out in hypertonic minimal medium to prevent bursting

of spheroplasts and cross feeding. One of the mutagenic treatments outlined above was employed.

2) The culture was grown overnight in minimal medium. 5mls was pipetted into a 250ml flask, 1 drop EMS added and the flask shaken for 2 hours at 37°. 45mls minimum medium containing the desired growth factor was added and incubation continued until a fully grown culture was obtained. The treatment with EMS was repeated. 10mls of the culture was removed, washed twice with minimal medium and suspended in 50mls minimal medium. Incubation was carried out with shaking for 1 hour at 37°. Penicillin was added (2,000 i.u/ml) and incubation continued for a further hour. It was stopped at the first sign of lysis. The culture was centrifuged and washed, then suspended in 45mls minimal medium containing the growth factor and incubated until fully grown. After washing the cells, the organisms were resuspended in 10mls minimal medium and plated out on minimal medium containing the growth factor. Replication was carried out on to appropriate media when the colonies were of suitable size.

CENTRIFUGATION

Small volume of cultures were centrifuged in a MSE Minor bench centrifuge at a dial setting corresponding to 2,000g. Otherwise centrifugation was carried out in a MSE High Speed 18 or MSE superspeed 40 centrifuge. Angle heads were generally used and the centrifugal force quoted is the maximum obtained at the tip of the tube. Volumes over 20 l were centrifuged using a continuous action rotor head.

PREPARATION AND PURIFICATION OF EXTRACELLULAR POLYSACCHARIDES

These were isolated and purified by the techniques described by Wilkinson,

Dudman and Aspinall (1955) and Dudman and Wilkinson (1956). The cells were harvested and suspended in saline containing 1% formaldehyde. If the strain was capsulate, the cell suspension was boiled for a few minutes to strip off the capsule or was treated with NaOH (1%^{W}/v) for 30 minutes with stirring, then neutralized with N HCl. The cells were deposited by centrifugation at 27,000g for 1 hour. The polysaccharide was precipitated from the supernatant by the addition of 2-3 volumes of ice-cold acetone. The precipitated material was washed with acetone and ether and dried. It was dissolved to a concentration of 0.5% in acetate buffer containing 4% sodium acetate and 2% acetic acid. Each 100ml portion was deproteinized by shaking with 4 or 5 successive aliquots of a mixture of 20mls chloroform and 4mls butanol. The aqueous solutions were combined, centrifuged to remove any precipitate, and dialyzed against running tap water for 4-5 days. The solution was concentrated under reduced pressure and freeze-dried. These polysaccharide preparations were kept at room temperature in air tight containers.

PREPARATION AND PURIFICATION OF LIPOPOLYSACCHARIDES

The cells were harvested by centrifugation and freeze-dried. Lipopolysaccharide was extracted by the water/phenol method of Lüderitz *et al* (1965). After dialysis the aqueous layer was concentrated under reduced pressure. The lipopolysaccharide was deposited by ultracentrifugation at 100,000g for 4 hours, then freeze-dried. The supernatant fluid was freeze-dried and assayed for glucose as a measure of glycogen production.

Preparation of polysaccharide free from lipid was accomplished by the method of Davies (1955). A 1% (^{W}/v) solution of lipopolysaccharide in 1%

($^{v}/v$) acetic acid was hydrolysed at 100° in a sealed ampoule for 1 hour. The lipid was deposited by centrifugation. The supernatant fluid was dialysed overnight against running tap water and freeze-dried.

PREPARATION AND PURIFICATION OF NUCLEOTIDES

The cells were harvested by centrifugation and washed with ice-cold saline. The nucleotides were extracted by the method of Morikawa, Imae and Nikaido (1964). Ethanol was added to the cell suspension to make a final concentration of 70% and the solution was heated at 70° for 10 minutes with stirring. The cell debris was removed by centrifugation at 10,000g and re-extracted with ethanol. The supernatants were combined and concentrated under reduced pressure to a small volume. Protein was removed by shaking with an equal volume of chloroform for 5 minutes and centrifuging at 11,000g for 5 minutes. The aqueous layer was collected, shaken again with chloroform and centrifuged. At this stage the aqueous layer was very cloudy; centrifugation at 18,000g for 30 minutes cleared the emulsion. The solution was stored at -20°.

Column chromatography of this solution was carried out on Whatman ET 11 Ecteola cellulose by the method of Nilsson and Sjunnesson (1961), as modified by Grant (1968). The cellulose powder was suspended in 0.5N NaOH under vacuum for 30 minutes. It was washed on a glass pad under suction with water until the washings were neutral, then was suspended in 1.0M triethyl ammonium acetate buffer pH 4.0. It was degassed under pressure again for 30 minutes. A column (25 x 1cm) was packed with the powder under gravity and equilibrated overnight with the same buffer at a flow rate of 20mls/hr. Distilled water was washed through the column for the next 24 hours. The nucleotide extract was absorbed on the column. The effluent was continuously monitored at 254mμ and recorded

(LKB 4701A Uvicord I, and recorder, LKB Instruments Ltd., London). The column was washed through with water to elute any uncharged uv absorbing material. When absorption returned to zero a buffer gradient was started comprising 500mls distilled water in the mixing vessel and 0.5M triethyl ammonium acetate pH 6.0 in the reservoir vessel. 10ml fractions were collected until all the uv absorbing material had been eluted. The tubes containing these materials were pooled and freeze-dried.

HYDROLYSIS OF EXTRACELLULAR POLYSACCHARIDES AND LIPOPOLYSACCHARIDES

Extracellular polysaccharides were completely hydrolysed by heating with 2N H_2SO_4 in sealed tubes for 16 hours at 100°. Lipopolysaccharides were hydrolysed by heating with N H_2SO_4 for 8 hours at 100° in sealed tubes.

The solutions were diluted with distilled water and neutralized by use of Amberlite IRA-410 (HCO_3^- form) resin. After removal of the resin the hydrolysates were concentrated to small volumes.

PAPER CHROMATOGRAPHY AND ELECTROPHORESIS

Whatman No. 1 paper was used.

a) Solvents

I. Pyridine: ethyl acetate: acetic acid: water 5 : 5 : 1 : 3 (v/v)
(Fischer and Dörfel, 1955)

II. Ethyl acetate : acetic acid : formic acid : water 18: 3 : 1 : 4 (v/v)
(Feather and Whistler, 1962).

III. Ethyl acetate : pyridine : water 10 : 4 : 3 (v/v) (Gould, 1965)

IV. 1.0M ammonium acetate : ethanol 3 : 7 (v/v) pH 7.5.
(Paladini and Leloir, 1952)

Irrigation times were 20 hours, 40 hours, 12 hours and 16 hours respectively.

b) Electrophoresis

Buffer - pyridine : acetic acid : water 5 : 2: 43 (v/v) pH 5.3

(Sutherland, Lüderitz and Westphal, 1965).

A Locarte high-voltage paper electrophoresis equipment was used. Separation of charged oligosaccharides was obtained in 4 - 5 hours using 80- 100 mamp and salts were removed in 30-40 minutes.

Sugars were detected with alkaline Ag NO_3 reagent (Trevelyan, Proctor and Harrison, 1950)

ULTRASONIC DISINTEGRATION AND FRACTIONATION OF CELL CONTENTS

Cells were washed with cold saline and resuspended in 0.1M Tris-HCl buffer pH 8.0. They were disrupted for periods of 90 seconds in 5 - 20ml batches, cooled in crushed ice. An MSE 100 watt ultrasonic disintegrator automatically tuned to 20Kc/sec was used. Whole cells were removed by centrifuging twice at 2,500g for 15 minutes. The supernatant was used as a crude enzyme fraction. The cell wall-membrane fraction was deposited by centrifugation at 26,000g for 60 minutes. It was suspended in a small quantity of ice cold water.

WASHED CELL SUSPENSIONS

The method of Wilkinson and Stark (1956) was used.

The cells were harvested by centrifugation, washed in saline and resuspended in 0.1M phosphate buffer pH 7.0. 250ml Erlenmeyer flasks contained 25mls 0.1M phosphate buffer pH 7.0, 5mls 0.5%KCl, 10mls glucose (100mg/ml), a trace of $MgSO_4$, and the cell suspension. Incubation was carried out on shaking water baths. At intervals 5ml samples were removed, formalized, and in the case of

capsulate organisms boiled for a few minutes. The cells were deposited by prolonged centrifugation and resuspended in 5mls saline. Both supernatant and cell suspension were dialyzed against running tap water for 2-3 days. The volumes of the sacs were measured after dialysis. Polysaccharide production was determined by the anthrone method (Fairbairn, 1953), cellular protein by the method of Lowry, Rosebrough, Farr and Randall (1951), and cellular nitrogen by the micro-Kjeldahl method.

ANALYTICAL METHODS

1) Spectrophotometry.

All measurements were carried out in a Unicam SP 500 instrument (Unicam Instruments Ltd., York Street, Cambridge). Silica and glass micro- and glass semi-micro cells were used.

2) Counting of radioactivity

An automatic ambient temperature liquid scintillation counter with automatic print-out was used (Beckman Instruments Ltd., Glenrothes, Scotland). Aqueous samples were counted in a scintillator consisting of NE 572 "scinstat" dissolved in dioxane (Nuclear Enterprises (G.B.) Ltd., Edinburgh). Non-aqueous samples were counted in a scintillator containing 2 parts AR toluene to 1 part Triton X-100 with 0.5% (W/v) 2,5-diphenyloxazole (PPO) and 0.013% (W/v) 1,4 - bis [2- (4- methyl -5-phenyloxazoly-1)] benzene (dimethyl POPOP). Efficiency of counting for C^{14} in these systems is about 90%. Radioactivity on paper was estimated by cutting into strips, generally 2 x 1cm, and immersing these in the dioxane-based scintillator or the toluene-based scintillator without Triton X-100. Counting efficiency for C^{14} is about 50%.

A tracerlab 4 Pi scanner was occasionally used to scan paper chromatograms.

3) Assay methods.

All glassware was cleaned with concentrated HNO_3 and glass distilled water. Micro adaptations of these following methods were used and constriction pipettes (H.E. Pedersen, Copenhagen, Denmark) were employed throughout:

a) Total polysaccharide was determined by the anthrone method (Fairbairn, 1953).

b) Glucose was determined by the use of glucose oxidase reagent (C.F. Boehringer und Soehne, G.m.b.h., Mannheim, Germany).

c) Galactose was determined by the use of galactostat (galactose oxidase) reagent (Worthington Biochemical Corp., Freehold, New Jersey, U.S.) or galactose dehydrogenase reagent (Boehringer).

d) Fucose was estimated by the method of Dische and Shettles (1951)

e) Glucuronic acid was estimated by the method of Bowness (1957)

f) Protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951)

g) Phosphorus was determined by the method of Fiske and SubbaRow (1925)

h) Total nitrogen was determined by the micro-Kjeldahl method using boric acid to trap the ammonia produced and estimating it colorimetrically with Nessler reagent.

The following enzyme assays were performed at room temperature in silica micro-cells by following the change in absorption at 340 mμ. Sufficient crude enzyme preparation was used to give a linear change in absorption over the first few minutes.

a) Hexokinase

Assay by modification of method of Slein, Cori and Cori (1950)

Assay mixture : 100 µl. 1.0 M Tris-HCl pH 7.5; 100 µl. 0.1 M cysteine-NaOH pH 8.5; 100 µl. H_2O; 30 µl. 1.0M $MgCl_2$; 20 µl. 0.1 M ATP; 20 µl. 0.02 M NADP; 30 µl. 0.2 M glucose; 1 µl. glc-6-P dehydrogenase (0.14 units)

b) Phosphoglucose isomerase

Assay by modification of method of Slein (1955)

Assay mixture : 100 µl. 1.0 M Tris-HCl pH 7.5; 100 µl. 0.1 M cysteine-NaOH pH 8.5; 100 µl. H_2O; 10 µl. 1.0 M $MgCl_2$; 20 µl. 0.1 M Fru-6-Ⓟ; 20 µl. 0.02 M NADP; 1 µl. Glc-6-P dehydrogenase (0.14 units)

c) Phosphomannose isomerase

Assay by modification of method of Slein (1955)

Assay mixture : 100 µl. 1.0 M Tri-HCl pH 7.5; 100 µl. 0.1 M cysteine-NaOH pH 8.5; 100 µl. H_2O; 10 µl. 1.0 M $MgCl_2$; 20 µl. 0.1 M Man-6-Ⓟ; 20 µl. 0.02 M NADP; 1 µl. glc-6-P dehydrogenase (0.14 units)

d) Glucose-6-phosphate dehydrogenase

Assay by modification of method of DeMoss (1955)

Assay mixture : 100 µl. 1.0 M Tris-HCl pH 7.5; 100 µl. 0.1 M cysteine-NaOH pH 8.5; 100 µl. H_2O; 10 µl. 1.0 M $MgCl_2$; 20 µl. 0.02 M NADP; 20 µl. 0.1 M Glc-6-Ⓟ

e) UDPG pyrophosphorylase

Assay by modification of method of Munch-Peterson and Kalckar (1955)

Assay mixture : 100 µl. 1.0 M Tris-HCl pH 7.5; 150 µl. H_2O; 5 µl. 1.0 M $MgCl_2$; 20 µl. 0.02 M NADP; 30 µl. 0.05 M $NaPP_i$; 20 µl. 0.02 M UDPG; 1 µl. Glc-6-P dehydrogenase (0.14 units); 2 µl. phosphoglucomutase (0.065 units)

f) GDPM pyrophosphorylase

Assay by modification of Munch-Peterson (1962).

Assay mixture: 100 µl. 1.0 M Tris-HCl pH 7.5; 100 µl. H_2O; 50 µl. 0.5 M NaF; 10 µl. 0.005 M ADP; 10 µl. 0.2 M glucose; 5 µl 1. 0 M $MgCl_2$; 20 µl. 0.02 M NADP; 20 µl. 0.05 M $NaPP_i$; 20 µl. 0.01 M GDPM; 2 µl hexokinase (0.54 units); 1 µl. Glc-6-P dehydrogenase (0.14 units)

g) GDPfucose synthetase

Assay by modification of method of Ginsburg (1966)

Assay mixture: 100 µl. 1.0 M Tris-HCl pH 8.0; 100 µl. H_2O; 10 µl. 0.02 M NADPH; 20 µl. 0.01 M GDPM

h) UDPG Dehydrogenase

Assay by modification of method of Strominger, Kalckar, Axelrod and Maxwell (1954)

Assay mixture: 50 µl. glycine - NaOH pH 8.7; 200 µl. H_2O; 5 µl. 1.0 M $MgCl_2$; 20 µl. 0.02 M NAD; 20 µl. 0.02 M UDPG.

i) UDPGal -4-epimerase

Assay by modification of method of Maxwell, Kurahashi and Kalckar (1962)

Assay mixture: 50 µl. glycine-NaOH pH 8.7; 150 µl. H_2O; 5 µl. 1.0 M $MgCl_2$; 20 µl. 0.02 M NAD; 20 µl. 0.0045 M UDPGal; 2 µl. UDPG dehydrogenase (1 unit)

Plate 1. *K. aerogenes* A1 grown on yeast extract medium at 30° for 48 hours

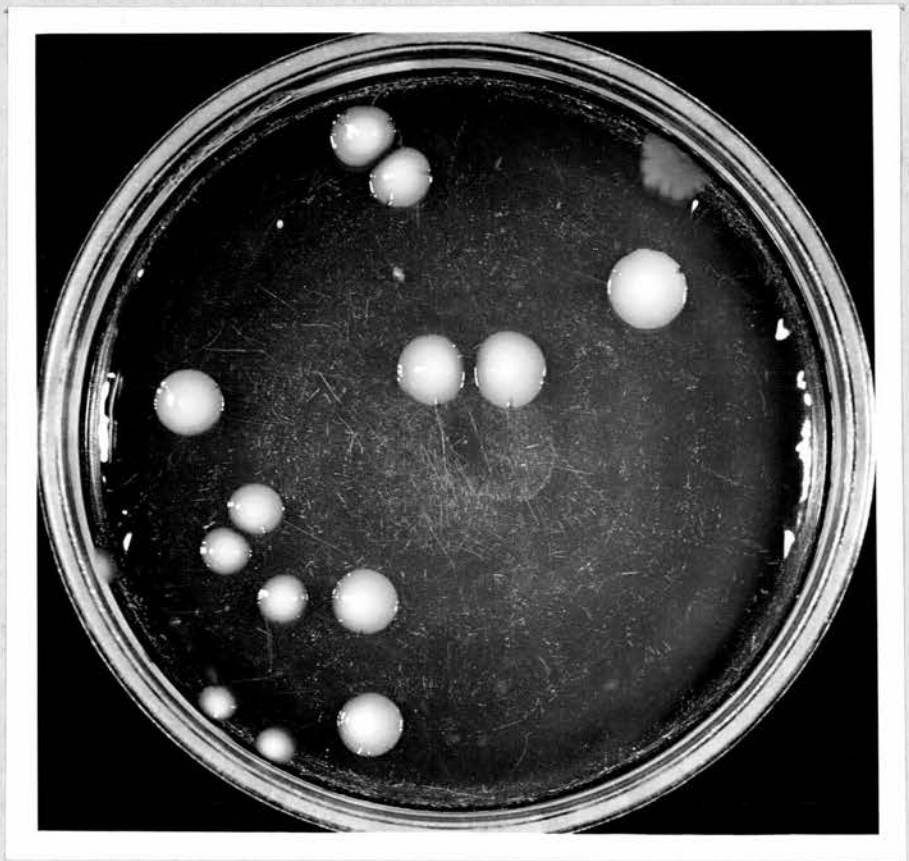


Plate 2. *K. aerogenes* A4 grown on yeast extract medium at 30° for 48 hours

RESULTS

SECTION A.

CULTURAL CHARACTERISTICS OF ORGANISMS

The synthesis of extracellular polysaccharides leads to the formation of a colony showing typical mucoid characteristics being domed in shape and of a smooth glistening appearance. K. aerogenes A1 and A4, particularly on media deficient in nitrogen, produced large raised shiny colonies of viscous consistency and regular outline. Their colony appearance on yeast extract medium is shown in Plates 1 and 2. On other media such as nutrient agar, polysaccharide synthesis was not so marked. The colonies were reduced in size and less raised. In liquid media the two strains were turbid and viscous due to polysaccharide production. Microscopic examination by the India ink method of Duguid (1951) showed both strains to be capsulate. K. aerogenes A1 formed very large capsules while those produced by A4 were smaller, their size varying according to the growth conditions. In addition cells of A1 after growth in liquid media proved to be more difficult to deposit by centrifugation due to the larger capsule and increased viscosity of the medium.

It had been noticed that occasionally a spontaneous mutant colony form arose which had lost the mucoid characteristics of the parent strain and which did not synthesize extracellular polysaccharide. Such a non-mucoid strain of K. aerogenes A3 has been described by Wilkinson, Duguid and Edmunds (1954). Analogous mutants of both A1 and A4 strains occurred. They arose spontaneously or more frequently if a mutagen was used. Their cultural characteristics were quite different from those of the parents. The colony of non-mucoid mutants of A1 was smooth and flat with an irregular, spreading outline; that of

Plate 3 K. aerogenes A4(0) grown on yeast extract medium at 30° for 48 hours

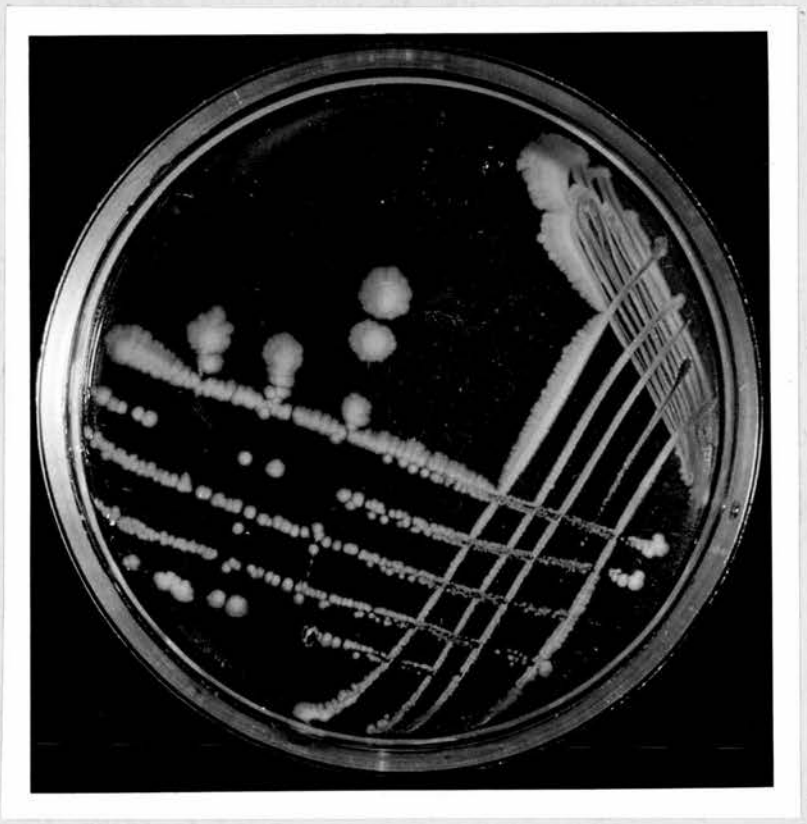


Plate 4 K. aerogenes A1(0) grown on yeast extract medium at 30° for 48 hours

non-mucoid mutants of A4 was more regular in outline, slightly smaller than the parent in size but non-mucoid and flat. Colonies of A4(0) and A1(0) mutants are shown in Plates 3 and 4. In liquid culture they grew with uniform turbidity and the viscosity of the medium did not increase. No capsules were distinguished in India ink preparations.

In addition to these mutants which did not produce any extracellular polysaccharide, a further mutant type of A1 was found synthesizing exopolysaccharide in a different manner from the parent. It arose on two occasions, one using MNG as mutagen and the other using ultra-violet light. The colony form was like that of A1 except that on prolonged incubation, such as five days at 30°, polysaccharide production was very extensive. Mucoid material tended to spread over the surface of the plate and to drop on the lid. The polysaccharide was found to be highly viscous and could be pulled out into a thread when tested with a loop. India ink films of surface cultures show that the mutant was not capsulate but that the polysaccharide material was present as amorphous slime. In liquid culture the production of this slime made the medium extremely viscous. The cells may be deposited by prolonged centrifugation leaving the polysaccharide dispersed in the supernatant. This mutant of A1 was called A1S1. An analogous mutant of A3 has been described (Wilkinson, Duguid and Edmunds, 1954). A plate culture of A1S1 on yeast extract medium is shown in Plate 5. Attempts to isolate a slime-producing mutant of A4 during the course of this work were unsuccessful.

Although polysaccharide was formed by K. aerogenes A1 and A4 on all media it was produced to the greatest extent on nitrogen-deficient carbon-rich media such as yeast extract medium. The incubation temperature did not make much difference. Polysaccharide was synthesized at all temperatures between

Plate 5 *K. aerogenes* A1S1 grown on yeast extract medium at 30° for 48 hours

15° and 37° although, of course, rate of growth and rate of polysaccharide production varied at the different temperatures. Normally when polysaccharide was required in greatest quantity, incubation for 3-4 days at a temperature of 30°-35° was employed.

EXOPOLYSACCHARIDE SYNTHESIS

a) ANALYSIS OF EXOPOLYSACCHARIDES

The polysaccharide of K. aerogenes A1 and A4 and that of the mutant A1S1 were prepared and purified. A portion of each was hydrolysed and analysed by paper chromatography in Solvents I, II and III. Sugars were identified on the basis of their mobilities in these solvents compared to standard sugars. The sugar components were analysed quantitatively and their ratio in the polysaccharide found. The results are shown in Table 3. Acetyl and pyruvyl groups were absent in both strains (I.W. Sutherland, unpublished).

Strain A1

Previously, using different analysis techniques, the exopolysaccharide of A1 had been shown to consist of 9-10% fucose, 47-48% glucose and 29-30% glucuronic acid (Dudman and Wilkinson, 1956). It thus contained the same monosaccharide components in the same proportions as A3 and both belonged to Type 54 antigenically. However differences in chemical structure between the two strains were shown as a result of partial acid hydrolysis (Gould and Sutherland, unpublished) and hydrolysis using phage depolymerases (Sutherland, 1967). The polysaccharide of A3S1 was re-analysed by Sandford and Conrad (1966) and was found to consist of the sugars, glucose, fucose and glucuronic acid in the ratio 2: 1: 1. A repeating unit consisting of a tetrasaccharide was suggested (see Introduction, Section B, p 25). However the polysaccharide of A3 was O-acetylated and results from depolymerase experiments indicated that the re-

TABLE 3.

The Composition of Slime and Capsule Exopolysaccharides

Strain	Constituents	Molar ratio
A1	glc : glUA : fuc	2 : 0.90 : 1.10
A1S1	glc : glUA : fuc	2 : 1.0 : 1.06
A4	glc : gal : glUA	1 : 2.2 : 0.97

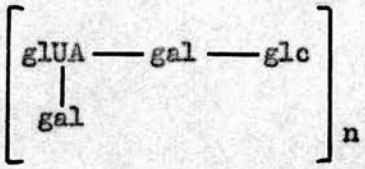
peating unit was more likely to be an octasaccharide containing glucose, glucuronic acid, fucose and acetyl in the ration 4: 2: 2: 1 with an unknown component also present (Sutherland, 1967). The polysaccharide of A1 was found here to contain glucose, glucuronic acid and fucose in the ratio 2: 1: 1, but without acetyl groups. The order of monosaccharide units and their linkages have not been completely determined but two aldobiuronic acids i) a β-glucuronosyl-fucose and ii) a β-glucuronosyl-glucose have been identified in partial acid hydrolysates.

The polysaccharides of A1 and A1S1 consisted of the same monosaccharides in the same molar proportions. Galactose had been reported in the polysaccharide of A1 at a concentration of 1% (Dudman and Wilkinson, 1956). This was thought to be due to contamination by lipopolysaccharide (Sutherland and Wilkinson, 1966). This conclusion was confirmed as no galactose was found in the preparation of the polysaccharide of A1 or A1S1 either chromatographically or using galactose oxidase after hydrolysis.

Strain A4

Previously the polysaccharide of A4 had been shown to consist of 22% glucose, 51% galactose, 25% glucuronic acid and 3% mannose (Dudman and Wilkinson, 1956). The polysaccharide used for the present study contained glucose, galactose and glucuronic acid in the ratio 1: 2: 1. No mannose was detected chromatographically. Partial hydrolysis of the polysaccharide with $\underline{N}$ H_2SO_2 for 15 minutes at 100° followed by electrophoresis showed the presence of three charged fractions and neutral material. All three charged fractions moved more slowly than glucuronic acid. They had mobilities on electrophoresis of 0.69, 0.53 and 0.42 with respect to glucuronic acid. The fastest moving was found by chemical analysis to be a disaccharide consisting of the sugars glucuronic acid and galactose. The second fastest moving was a

trisaccharide containing the sugars glucuronic acid, galactose and glucose. The slowest moving fraction has not been fully identified yet but may be a tetrasaccharide of galactose, glucose and glucuronic acid in the ration 2: 1: 1. From these results, the repeating unit of the polysaccharide was thought to consist of the tetrasaccharide:



(I.W. Sutherland, unpublished)

b) POLYSACCHARIDE SYNTHESIS DURING GROWTH

The polysaccharide content of the capsulate strain A1 during each phase of the growth curve was determined to find whether synthesis occurred linearly with growth and what quantities were synthesized by cells in log and stationary phase. Total polysaccharide production i.e. structural and internal polysaccharide synthesized as capsular material was measured during growth.

K. aerogenes A1 was grown overnight in nutrient broth at 30°. The cells were harvested by centrifugation, washed with saline and used to inoculate minimal medium. Incubation was carried out at 30° in a shaking water bath. At the beginning the capsules were small when the culture was examined microscopically using wet India ink films. After 3 hours incubation, most cells were in the process of division and small capsules were present. After 7 hours incubation the size of the capsules had enlarged greatly. At intervals samples were removed, fixed with formaldehyde and dialysed against running tap water for 2 days. Nitrogen was estimated by the micro-Kjeldahl technique and polysaccharide by the anthrone method. The amount of polysaccharide present/ mg cellular nitrogen was then calculated. The results are shown in Figs.15 and 16.

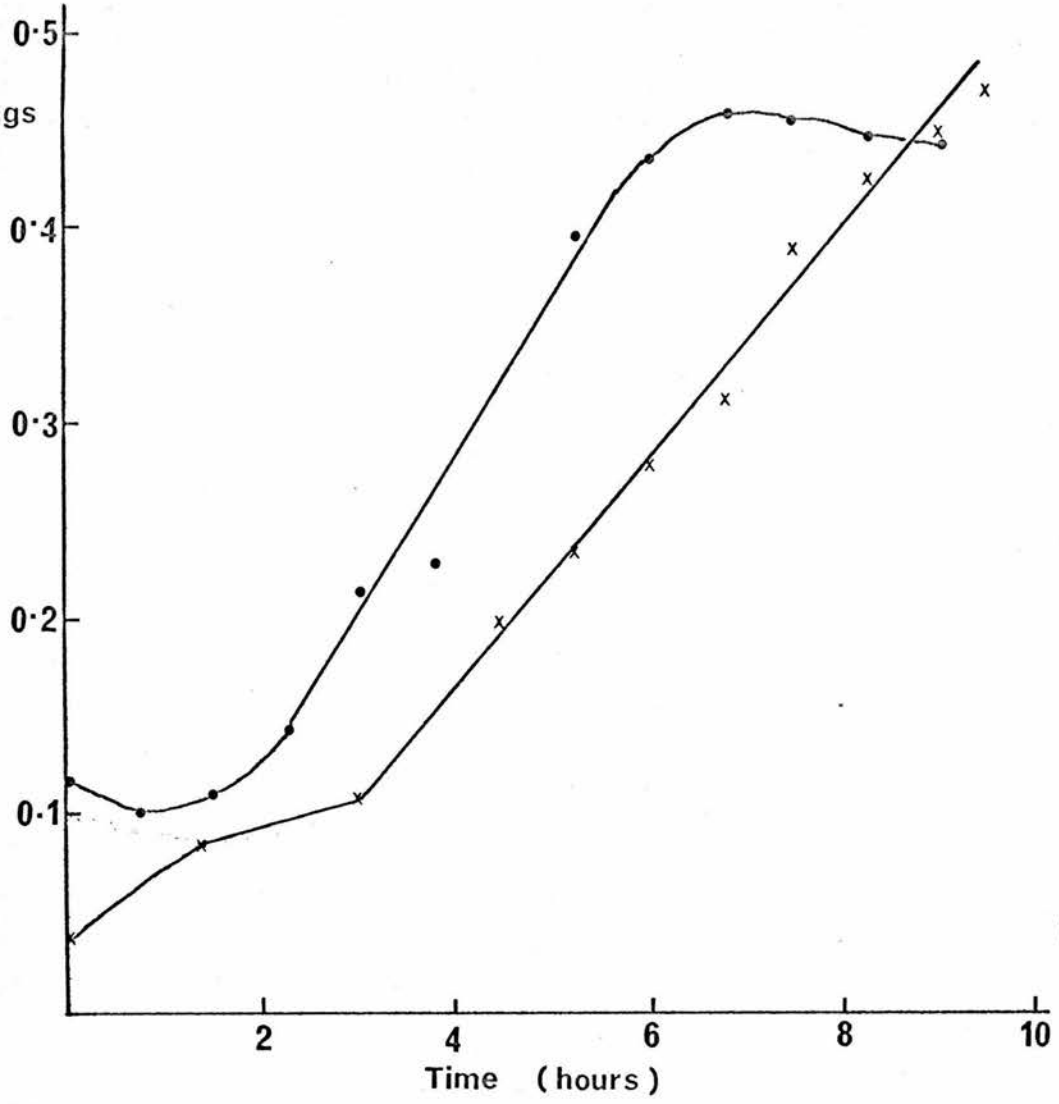


FIG 15. Quantity of polysaccharide (x—x) and cellular nitrogen (•—•) synthesised during incubation.

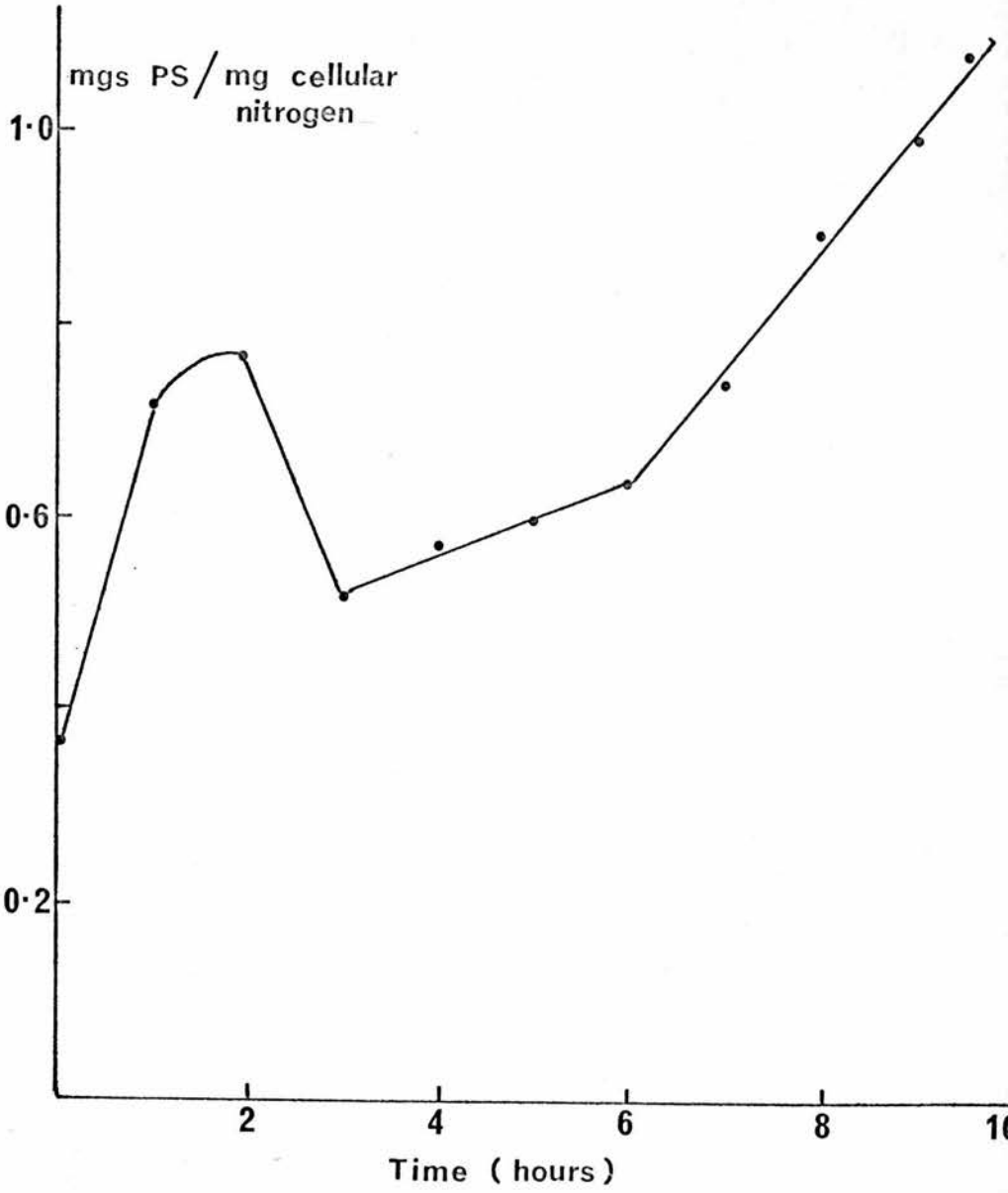


FIG 16. Mgs polysaccharide synthesised per mg cellular nitrogen during incubation.

From determinations of the nitrogen content of the culture it may be seen that a lag phase of about 1½ hours occurred before cellular growth started. Thereafter the culture entered the log phase of growth, which continued for a period of about 4 hours when stationary phase began. During log phase the nitrogen content of the culture doubled every hour. Total polysaccharide content increased as soon as the culture was put into the medium and continued throughout all phases of growth. The rate of polysaccharide synthesized /mg cellular nitrogen by log and stationary phase cells differed. For log phase it was 0.04mg/hour and for stationary phase cells 0.13mg/hour. Thus polysaccharide was formed three times more quickly in stationary phase cells. It is known that little glycogen if any is synthesized in log phase cells (Koeltzow, Epley and Conrad, 1968). Increased polysaccharide content of log cells was therefore due mainly to synthesis of extracellular polysaccharide and structural polysaccharide although perhaps it might be assumed that, in this phase, cellular nitrogen keeps in a fairly constant ratio with the latter. In stationary phase cells an enlargement of the capsule was observed microscopically and conditions were appropriate for synthesis of intracellular polysaccharide also. In the lag phase of growth there was increased carbohydrate content of the cells which diminished when the culture went into the log phase. This may reflect increased concentration of the intermediates of polysaccharide anabolism.

c) POLYSACCHARIDE SYNTHESIS BY LOG AND STATIONARY PHASE CELLS

Any difference in the ability of log and stationary phase cells of A1 to synthesize intracellular and extracellular polysaccharide was determined by use of washed cell suspensions. Cells in the log phase, 3 hours old, and stationary

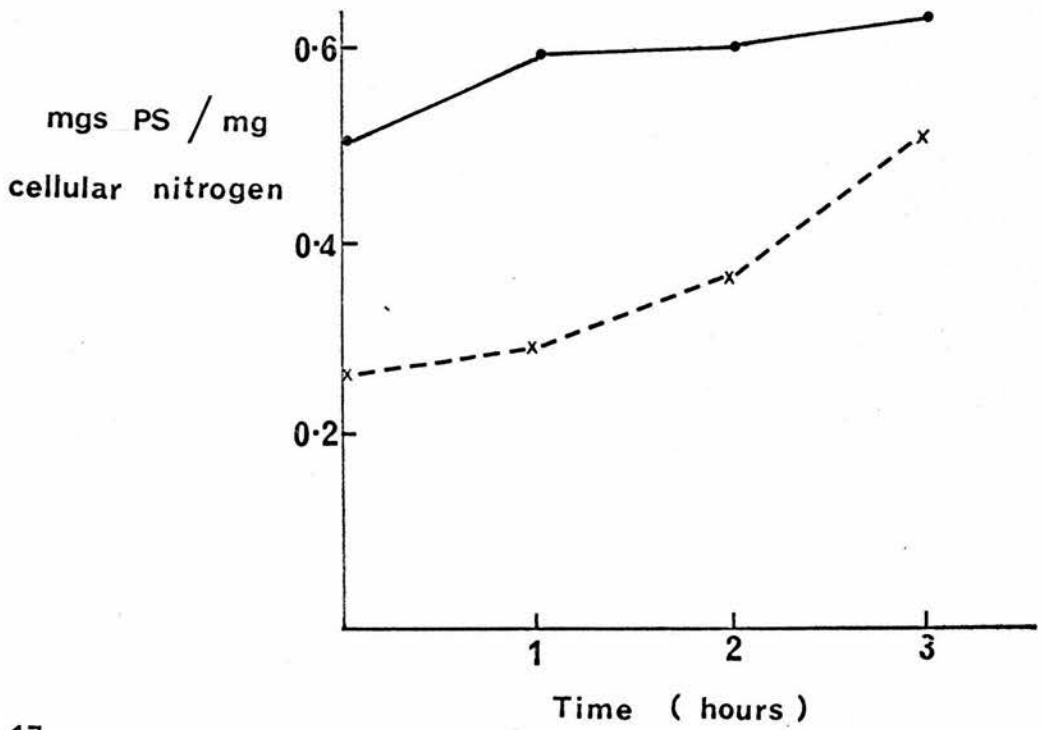


FIG 17

Quantity of polysaccharide synthesised per mg cellular nitrogen in washed cell suspensions of log (x--x) and stationary (•—•) phase cells of K. aerogenes A1.

phase, 16 hours old, were harvested, washed with saline and used in a washed cell experiment. Incubation was carried out at 30° for 3 hours. There was no external source of nitrogen so that the minimum synthesis of nucleic acid and proteins and cell division would occur. Most of the metabolic activity of the culture would then be directed towards the synthesis of polysaccharides. A short incubation time was used to ensure minimum autolysis of cells which would lead to the release of intracellular components. The nitrogen content of the cells increased by about 10% over the 3 hours incubation which indicated that some growth did occur and that polysaccharide production was not the only synthetic activity of the cells. Viable counts, performed by the method of Miles and Misra (1938) showed a constant level throughout the incubation period. Samples were removed at hourly intervals, dialysed and analysed as before to ascertain the amount of polysaccharide present per mg cellular nitrogen. The results are shown in Fig.17.

The cells in each phase were found to behave quite differently. Log phase cells at the start contained about half the amount of polysaccharide of stationary phase cells. There was a lag of about 1 hour before synthesis of polysaccharide started in the case of log phase cells, then it continued throughout the incubation period reaching the level of the stationary phase cells. The lag may be due to the build up of the intermediates essential for polysaccharide production. Stationary phase cells demonstrated no such lag and synthesis at a slow rate occurred over the whole incubation period. Possibly most of this was due to the synthesis of intracellular polysaccharide as the capsule may be at a maximum size in these cells, although some slime is always produced in addition to the capsule.

The A1S1 mutant of A1 was used so that a clear distinction might be made

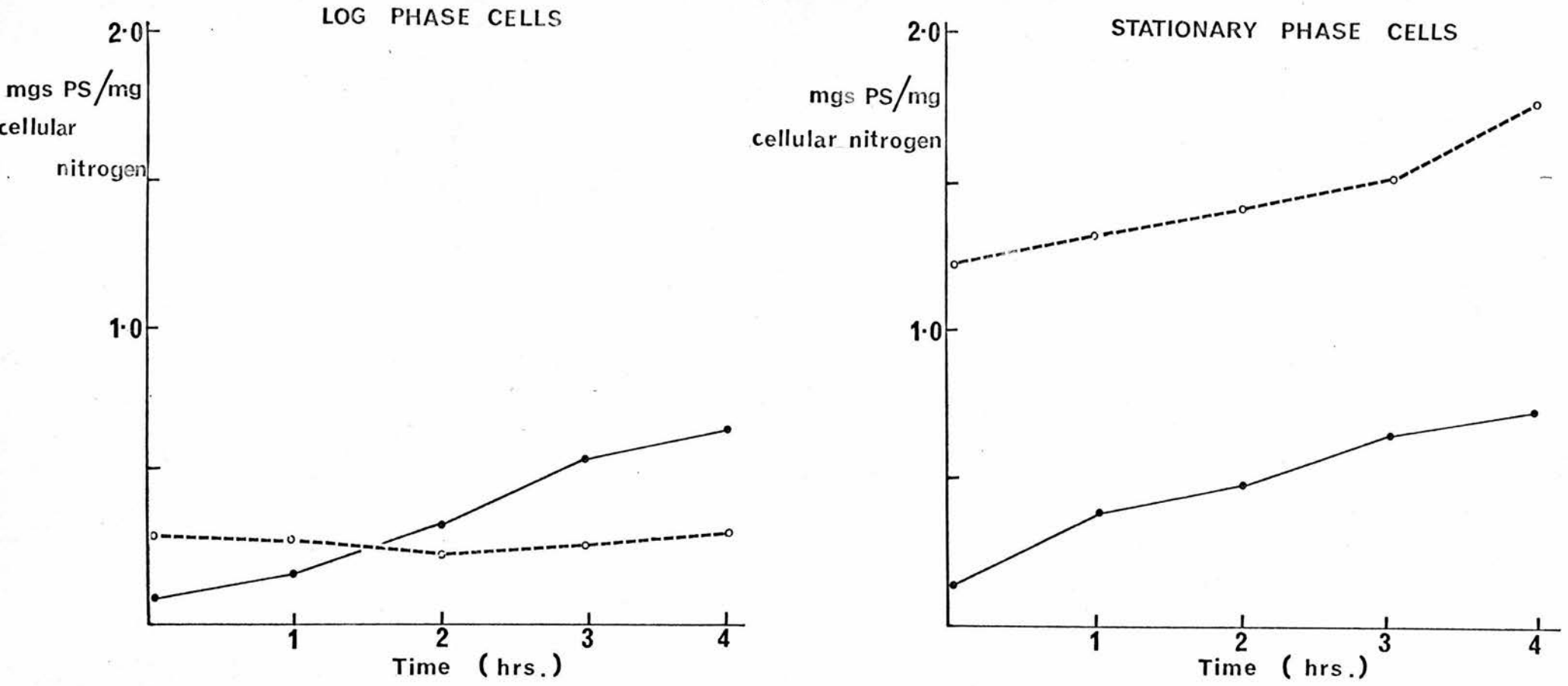


FIG 18

Amount of PS/mg cellular nitrogen synthesised intracellularily (∘– –∘) and extracellularily (•——•) by washed cell suspensions of A1SI

between intracellular and extracellular polysaccharide production. In the mutant the latter is synthesized as slime and, although a small proportion probably remains attached to the cells, most is released into the supernatant. A similar washed cell suspension experiment was carried out as with A1. The cells were separated from the extracellular polysaccharide in the supernatant by centrifugation before dialysis. The carbohydrate content of the supernatant and the cells were determined individually and intracellular and extracellular production / mg cellular nitrogen calculated for log and stationary phase cells. The results are given in Fig.18.

Log phase cells were shown to possess the ability to synthesize extracellular polysaccharide after a short lag period. This ability was shared by stationary phase cells to an equal extent. Log phase cells had a low content of intracellular polysaccharide and this level dropped slightly during the first half of the incubation period, probably to provide energy for synthesis of the intermediates for extracellular polysaccharide production. Stationary phase cells, on the other hand, had a high level of intracellular polysaccharide. This was not depleted during extracellular polysaccharide synthesis and increased still further throughout the incubation period.

Therefore as regards extracellular polysaccharide production by cells of A1S1, it appeared to make little difference if the cells were from the log or stationary phase.

d) POLYSACCHARIDE SYNTHESIS BY CELLS GROWN IN VARIOUS MEDIA

Log and stationary phase cells of A1S1 were prepared from cultures grown under varying conditions. The effect of these conditions on the subsequent synthesis of polysaccharide intracellularily and extracellularily was found.

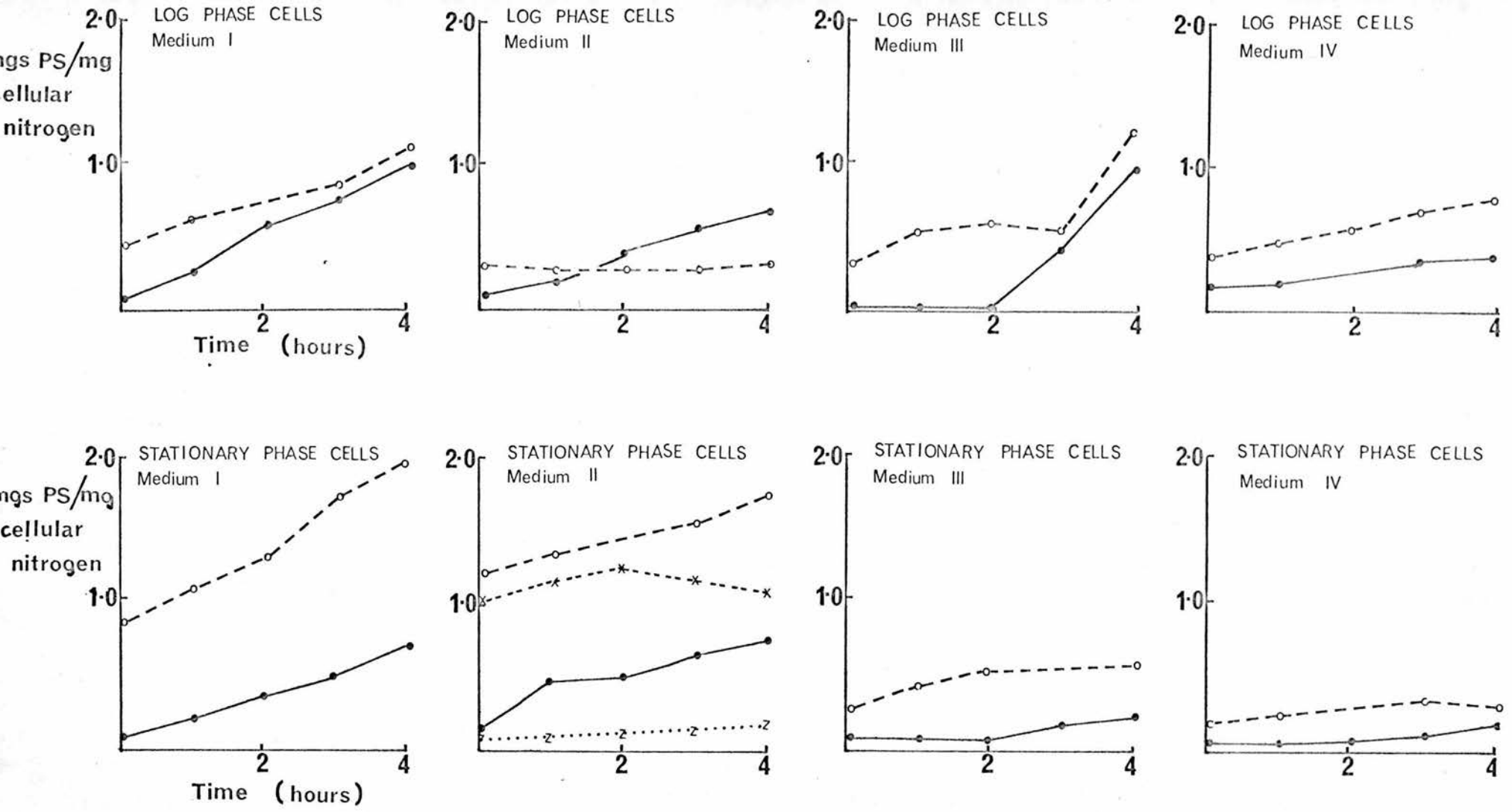


FIG 19.

Amount of PS / mg cellular nitrogen synthesised intracellularily (o—o) and extracellularily (•—•) by washed cell suspensions of log and stationary phase cells of AISI prepared by growth in Media I, II, III, and IV as indicated. Also 48 hour old cells grown in Medium II were used, and PS production intracellularily (x - - x) and extracellularily (z · · z) is shown.

The media used for the growth of the cells contained in all cases 1% $Na_2 HPO_4$, 0.3% $KH_2 PO_4$, 0.1% NaCl, 0.1% K_2SO_4, 0.02% $MgSO_4$. Added to this basic solution were:

I) 1% $(NH_4)_2 SO_4$, 1% glucose

II) 0.03% $(NH_4)_2 SO_4$, 1% glucose

III) 0.03% $(NH_4)_2 SO_4$, 1% citrate

IV) 2% casamino acids.

The cells were harvested after an incubation time of 3 hours for log phase cells and 16 hours for stationary phase cells. Cells were also grown for 48 hours in medium II. Washed cell suspensions were prepared and incubation started. The cells were separated from the supernatant by centrifugation before dialysis. Polysaccharide production / mg cellular nitrogen was measured. The results are shown in Fig.19.

Log phase cells grown in medium I possessed almost the same ability to synthesize extracellular polysaccharide as cells prepared from medium II. However if the glucose in the growth medium was replaced by citrate a lag of 2 hours occurred before exopolysaccharide synthesis started. Rate of synthesis by log phase cells grown in medium IV which has casamino acids as carbon and energy source was about half that of cells grown in medium I. There was no lag. In the cells harvested from the stationary phase, similar results were obtained.

Log phase cells grown in medium I were able to synthesize intracellular polysaccharide over the incubation period, while those from medium II did not. Rate of synthesis by cells grown in medium III was about half that of I. Cells prepared from medium IV showed a lag lasting about 3 hours before synthesis started. Stationary phase cells from media I and II showed a high level of

intracellular polysaccharide at the start of the incubation period. This level rose still further during incubation. Stationary phase cells from media III and IV had only low levels of intracellular polysaccharide and synthesis occurred at slow rates in both cases during incubation.

The 48 hour old cells grown in medium II showed little ability to synthesize polysaccharide either intracellularly or extracellularly when compared to 16 hour old or log phase cells.

It has therefore been shown that the medium in which the cells are grown may affect their subsequent ability to form intracellular and extracellular polysaccharide in washed cell suspensions. Log and stationary phase cells grown in a balanced medium containing glucose or in a nitrogen-deficient one also containing glucose were able to synthesize polysaccharide intracellularily or extracellularily. Old cells lost most of this ability. If citrate was used as carbon and energy source in the growth medium in place of glucose, there was a lag before synthesis as though the cells required to be adapted and intermediates necessary for polysaccharide production were not already present. Cells grown in a medium containing casamino acids as carbon and energy source showed only slight ability to synthesize polysaccharide material compared with the cells grown in other media.

e) EFFECT OF Ca^{++} AND Mg^{++} ON POLYSACCHARIDE PRODUCTION.

Wilkinson and Stark (1956) using washed cell suspensions of K. aerogenes A3S1 found the effect of various ions on polysaccharide production. K^{+} ions were required for synthesis of polysaccharide and Mg^{++} to a smaller extent. Ca^{++} stimulated extracellular polysaccharide production and phosphate ions were

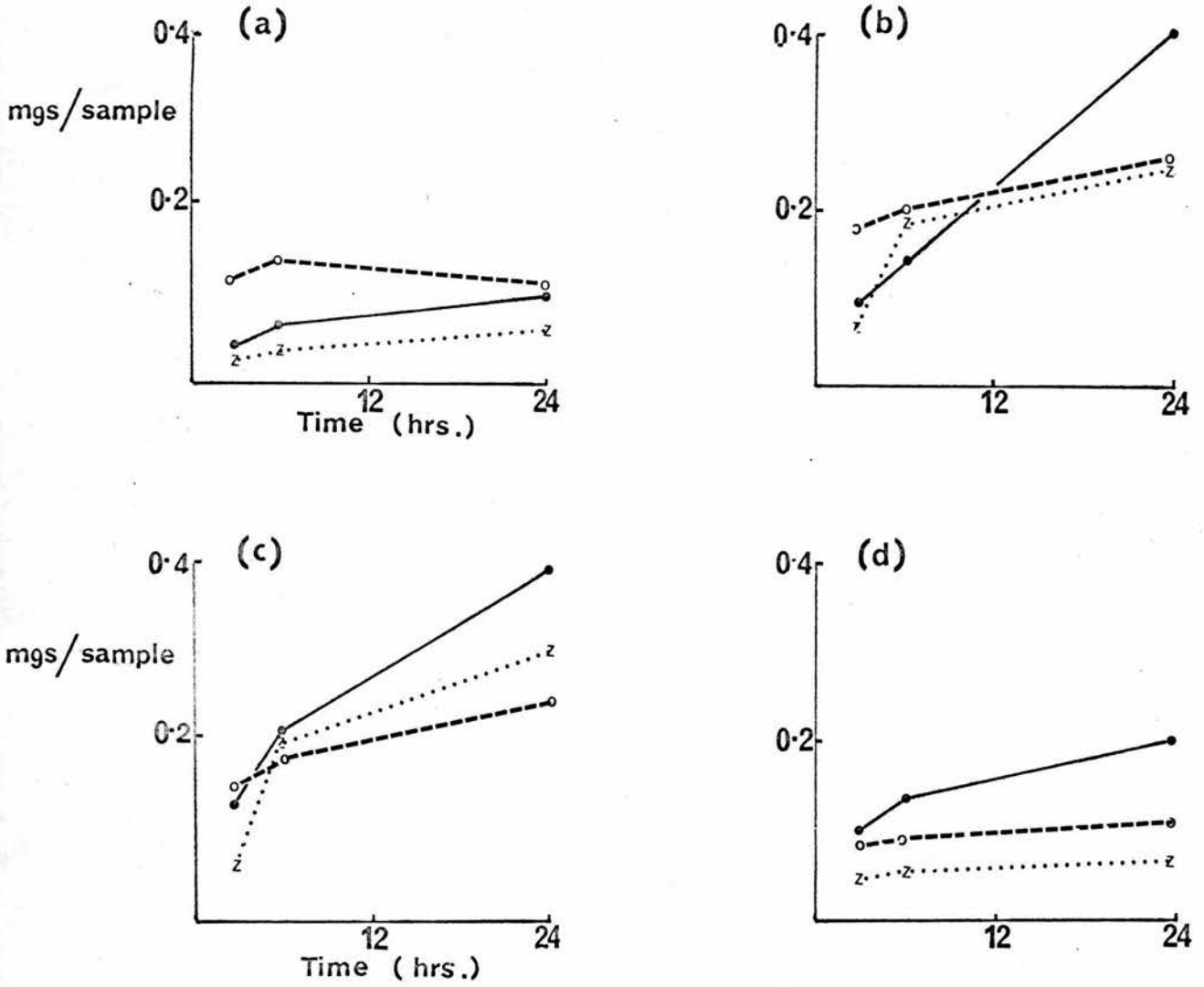


FIG 20

Amount of polysaccharide present extracellularily (●—●) and intracellularily (o--o) and cellular protein (z···z) during growth in media containing :

(a) no Ca^{++} or Mg^{++}
(b) Ca^{++} and Mg^{++}
(c) Mg^{++} alone
(d) Ca^{++} alone

inhibitory. It was decided to find out whether intracellular or extracellular production of polysaccharide in A1S1 would occur in a growth medium containing neither Ca^{++} nor Mg^{++} or containing only one of them.

Cells of A1S1 were grown at 30° in minimal medium for 16 hours. They were harvested and washed three times with saline under aseptic conditions. They were used to inoculate flasks containing 1% glucose, 1% $Na_2 HPO_4$, 0.3% $KH_2 PO_4$, 0.1% NaCl, 0.1% $K_2 SO_4$ and the following supplements:

I) none

II) 0.5 mM Ca^{++} as $CaCl_2$, 0.5 mM Mg^{++} as $Mg SO_4 . 7H_2O$

III) 1 mM Mg^{++}

IV) 1 mM Ca^{++}

Incubation was carried out at 30°. At intervals samples were removed and analysed after centrifugation and dialysis for the production of intracellular and extracellular polysaccharides and for protein which indicated growth. The results are shown in Fig.20.

In the absence of Ca^{++} and Mg^{++} little growth as demonstrated by the very small rise in cellular protein occurred. There was a small amount of extracellular polysaccharide produced and the level of intracellular polysaccharide fell over the 24 hour incubation period. Addition of Ca^{++}, although it did not affect growth, increased extracellular polysaccharide production by two-fold. Addition of both Ca^{++} and Mg^{++} to the medium allowed protein increase in a normal growth curve. The level of extracellular polysaccharide rose considerably during the inoculation period and there was also increase in intracellular polysaccharide level. Comparison of graphs (b) and (c) showed that Ca^{++} had only a small additive effect on polysaccharide production when Mg^{++} was already present.

The necessity for the presence of Mg^{++} to allow growth and polysaccharide

TABLE 4.

% labelling of fractions during incubation

Time (mins.)	1	5	10	25	90
Nucleotide pool	1%	1.3%	1.8%	3%	4.5%
Lipopolysaccharide	0.006%	0.03%	0.06%	0.32%	1.2%
Exopolysaccharide	2.1%	2.5%	2.6%	2.4%	4.3%

TABLE 5.

% labelling of components of exopolysaccharide

Time (mins)	glucose	fucose	glucuronic acid
10	96%	4%	0%
25	80%	17%	2%
90	41%	31%	28%

synthesis was therefore indicated. Mg^{++} cannot be replaced by Ca^{++}.

f) INCORPORATION OF C^{14} -Glc-6-Ⓟ INTO EXOPOLYSACCHARIDE.

Strain A1S1.

Labelled glc-6-Ⓟ was used to indicate whether the monosaccharide components of the extracellular polysaccharide were synthesized from glucose. To minimize the incorporation of the glc-6-Ⓟ into intermediates required for the growth of the cell, washed cell suspensions were employed. Under these conditions the bulk of the glc-6-Ⓟ taken up by the cells will be utilized to provide the intermediates for polysaccharide synthesis. Uptake into exopolysaccharide, nucleotide pool and lipopolysaccharide fractions were measured. The extracellular polysaccharide may be easily separated from the cells by centrifugation and the nucleotide pool may be extracted from the cell deposit by use of ethanol. Finally the lipopolysaccharide may be extracted from the cell debris by phenol-water treatment. Exopolysaccharide may be hydrolysed subsequently and % labelling of each sugar component found.

The cells were adapted to glc-6-Ⓟ by growth overnight at 30° in minimal medium containing 0.05% glc-6-Ⓟ in place of 2% glucose. The cells were harvested, washed and incubated at 30° for 90 minutes in the same medium as previously used for washed cell suspension experiments, except glucose was replaced by glc-6-Ⓟ. 0.05g carrier glc-6-Ⓟ was used together with C^{14}- glc-6-Ⓟ (1.1×10^7 d.p.m.). The cells were deposited by centrifugation. The extracellular polysaccharide was precipitated by pouring the supernatant into acetone. The precipitate was dried, dissolved in water, dialysed and lyophilized. It was redissolved in a small quantity of water. The cell deposit was extracted with 70% ethanol at 70°

for 10 minutes. The cell debris was deposited by centrifugation and the supernatant evaporated to a small volume under reduced pressure. The deposit was extracted with phenol-water, the water layer dialysed and ultracentrifuged to obtain the lipopolysaccharide. It was suspended in a small volume of water.

Total counts found in each fraction were :

exopolysaccharide	(% of total)	4.3%
lipopolysaccharide		1.2%
nucleotide pool		4.5%

A portion of the extracellular polysaccharide was completely hydrolysed. Chromatography was carried out in solvent I and the paper scanned in the Tracerlab 4 Pi scanner. A large peak corresponding to glucose occurred. Glucuronic acid and fucose were labelled to about half the extent of glucose.

The % labelling of the various fractions at intervals throughout the incubation period was found. The results are shown in Table 4.

The nucleotide fraction was thus labelled one minute after the addition of C^{14}-glc-6-Ⓟ. Labelling of this fraction increased during the 90 minute incubation period. The same was true of the lipopolysaccharide fraction. Labelling of the extracellular polysaccharide was 2.1% after only one minute incubation and stayed at the same level throughout the first 25 minutes of the experiment. It was thought that possibly the C^{14}-glc-6-Ⓟ had been enmeshed in the slime polysaccharide and was not entirely removed by dialysis. This may account for the unexpectedly high count after 1 minute.

The extracellular polysaccharide fractions at times 10, 25 and 90 minutes were hydrolysed and chromatographed in Solvent I. Sections corresponding to glucose, fucose and glucuronic acid were cut out and counted in the dioxane-

based scintillator. The % labelling of each component of the polysaccharide is shown in Table 5.

Labelling after 10 minutes incubation was shown to be almost entirely in the glucose component. After further incubation the fucose and glucuronic acid components of the polysaccharide were also labelled. Their % labelling increased with time while the % labelling of glucose dropped. Glc-6-P thus provided the carbon skeleton for the three components glucose, fucose and glucuronic acid of the polysaccharide. It was thought that at the start of the experiment, synthesis of the polysaccharide may occur using the nucleotide sugar precursors already present in the soluble pool without utilising C^{14}-Glc-6-P. The first sugar in the polysaccharide to be labelled was glucose indicating that the existing supply of UDPG had been finished while sufficient UDPGlUA and GDPfucose remained to allow synthesis of the polysaccharide without their being replenished from C^{14}-Glc-6-P. Later glc-6-P provided all three components of the polysaccharide.

Strain A4

Incorporation of C^{14}-glc-6-P into the various fractions of A4 was also determined. The experiment was carried out as for A1S1 except that incubation was continued for 120 minutes at 30°. At the finish the cell suspension was mixed in a small Waring blendor for 10 minutes to strip off the capsular material.

Total counts found in each fraction were :

exopolysaccharide	(% of total)	9%
lipopolysaccharide		0.27%
nucleotide pool		2%

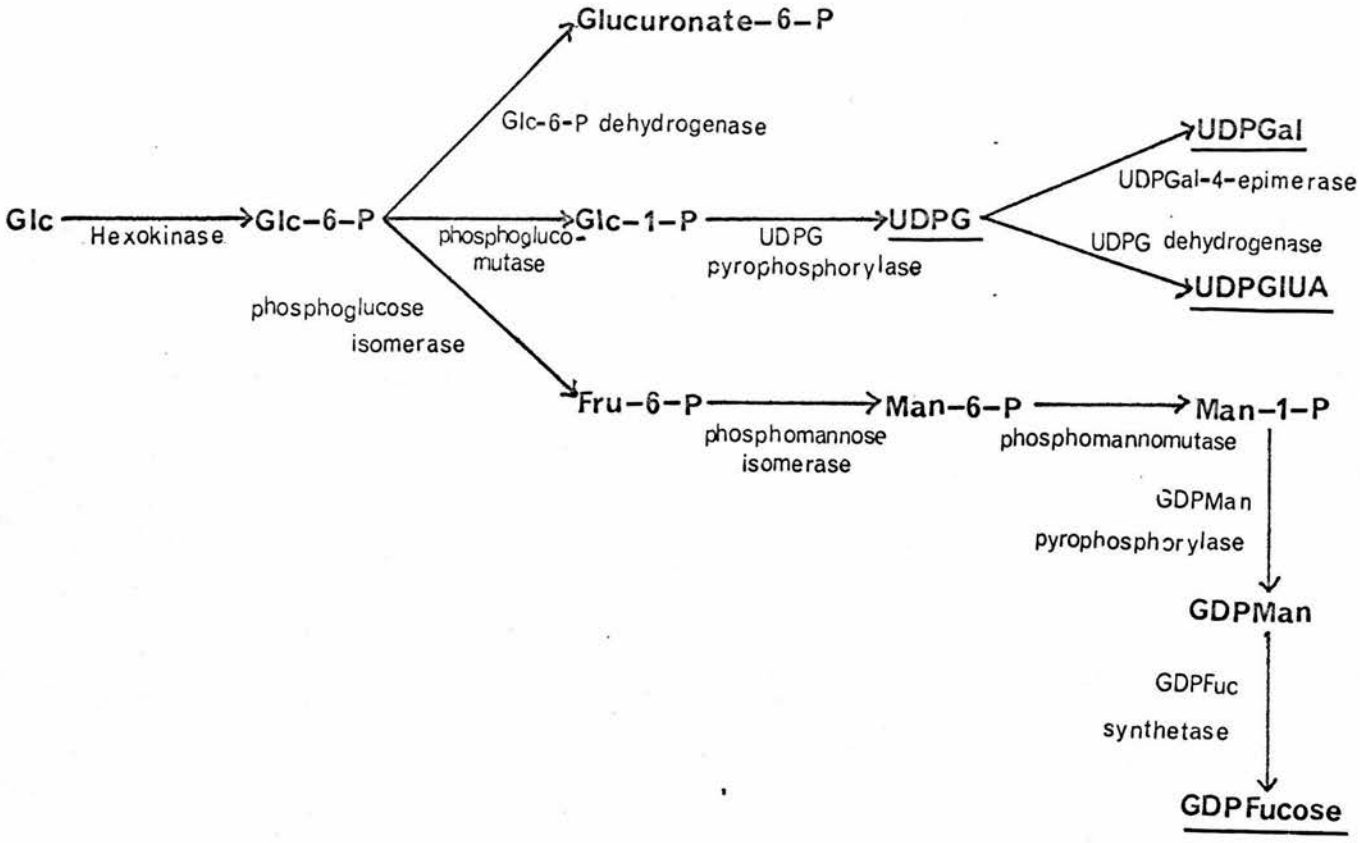


FIG 21 Pathways leading to the nucleotide sugar precursors of exopolysaccharides of K. aerogenes A1 and A4.

The polysaccharide was subsequently completely hydrolysed and chromatographed in Solvent III. The paper was scanned in the Tracerlab 4 Pi scanner. Peaks of radioactivity corresponding to glucose, galactose and glucuronic acid were obtained. These sections were cut out and counted in the dioxane-based scintillator. Glucose and galactose were labelled equally and glucuronic acid about half this level. Glc-6-P was thus used to synthesize all three components of the extracellular polysaccharide of strain A4.

ENZYME LEVELS

a) ENZYME LEVELS OF STRAINS A1, A1S1, and A4

Nucleotide sugars are thought to provide the precursors for the synthesis of exopolysaccharides. In K. aerogenes A4 the exopolysaccharide consists of the sugars glucose, galactose and glucuronic acid and the nucleotide sugars concerned are assumed to be UDPG, UDPGal and UDPGlUA. In A1 and A1S1 the polysaccharide consists of glucose, glucuronic acid and fucose, and the nucleotide sugar precursors are assumed to be UDPG, UDPGlUA and GDPfucose. The pathways and enzymes involved in the synthesis of these nucleotide sugars are shown in Fig.21.

Assay procedures were developed for nine of these enzymes - hexokinase, glc-6-P dehydrogenase, phosphoglucose isomerase, phosphomannose isomerase, UDPG pyrophophorylase, UDPGal-4-epimerase, GDPMan pyrophophorylase, UDPG dehydrogenase and GDPfucose synthetase. Enzyme activity was measured as units/mg protein where 1 unit equals the amount of enzyme catalysing transformation of 1 μmole substrate per minute. Protein was estimated by the method of Lowry et al (1951) using bovine serum albumin as standard.

TABLE 6.

Enzyme levels of various strains

	A1S1	A1	A4
Hexokinase	11.5	42	22.4
Glc-6-P dehydrogenase	115	90	16.2
phosphoglucose isomerase	90		7.6
Phosphomannose isomerase	19		3.4
UDPG pyrophosphorylase	28		16.8
GDPM pyrophosphorylase	4.3		0.02
GDPfucose synthetase	0.11	0.39	0
UDPG dehydrogenase	0.11	0.22	0.11
UDPGal-4-epimerase	0.06		0.84

Assays for the first six enzymes mentioned above proved satisfactory. The procedure for GDPMan pyrophosphorylase depended on the presence of two additional enzymes, phosphomannose isomerase and phosphomannomutase. The specific activity of the pyrophophorylase is thus difficult to obtain accurately. The assay for GDPfucose synthetase depended on the reduction by NADPH of the intermediate GDP-4-keto-D-rhamnose formed from GDPMan. In a few instances this was overshadowed by the non-specific oxidation of NADPH. If there was any doubt as to the presence of the enzyme, the appearance of fucose was determined chromatographically. In a similar way the assay procedure for UDPG dehydrogenase was affected by the non-specific reduction of NAD and the oxidation of NADH when formed. In a few cases where it was doubtful if the enzyme was present or not, labelled UDPG was used and the appearance of labelled UDPGlUA was determined chromatographically. Generally however a value for the specific activity was obtained using the assay as given.

Enzyme levels of cells of A1, A1S1 and A4 grown for 15 hours in yeast extract medium at 34° are shown in Table 6. Crude enzyme preparations from ultrasonicated cells were used. No attempt was made to fractionate the cell contents further. It proved difficult to work with extracts of A1 cells as the large amounts of capsular material present in the preparation made it impossible to deposit whole cells left after ultrasonic treatment. In addition, a high initial absorbance at 340mμ was created and the accuracy of the assays correspondingly decreased. A1S1 cells were used in preference to A1 as they could be obtained virtually free from extracellular polysaccharide material. Much less capsular polysaccharide was produced by cells of A4 and the difficulties encountered with A1 were not found to the same extent.

A striking feature of these enzyme levels was the large difference between

TABLE 7.

Enzyme levels of log and stationary phase cells of A1S1

	Log phase	Early stationary phase	Late stationary phase
Hexokinase	28	20.2	13.4
Glc-6-P dehydrogenase	78	69	49
phosphoglucose isomerase	95	98	56
phosphomannose isomerase	20.1	21.8	13.4
UDPG pyrophosphorylase	67	44.8	
GDPM pyrophosphorylase	0.9	0.56	0.45
UDPG dehydrogenase	0.28	0.28	0.28

the enzymes used for the normal metabolic activities of the cell such as glc-6-P dehydrogenase and phosphoglucose isomerase, and those used perhaps solely to synthesize the intermediates of the exopolysaccharide, such as UDPG dehydrogenase and GDPfucose synthetase. AlS1 possed the enzyme UDPGal-4-epimerase although the extracellular polysaccharide has no galactose component. It may, however, be required for synthesis of the lipopolysaccharide. A4 had no GDPfucose synthetase activity and little, if any GDPMan pyrophosphorylase activity. Fucose is not a monosaccharide component of the A4 capsule and may not be required within the cell at all. There are other considerable variations in the levels of equivalent enzymes in A4 and AlS1 strains reflecting the differences in polysaccharide metabolism of these strains.

b) ENZYME LEVELS OF LOG AND STATIONARY PHASE CELLS

Extracts of AlS1 cells from the log, early stationary and late stationary phases were assayed to find any difference in specific activities of the enzymes involved in synthesis of exopolysaccharide. The cultures were grown for 3 hours for log phase, 15 hours for early stationary phase and 48 hours for late stationary phase in minimal medium at 30°. The levels of the enzymes hexokinase, glc-6-P dehydrogenase, phosphoglucose isomerase, phosphomannose isomerase, UDPG pyrophosphorylase, GDPMan pyrophosphorylase and UDPG dehydrogenase were estimated. The results are shown in Table 7.

The levels of almost all the enzymes assayed dropped by about half in late stationary phase cells compared to log phase. It had been found previously, using washed cell suspensions, that the ability of late stationary phase cells to synthesize extracellular polysaccharide material was poor. Additional enzymes

may be required before synthesis of polysaccharide occurs such as a transferase for each sugar component and polymerase(s). The levels of these enzymes may also be affected by aging of the culture resulting in inability to synthesize the polysaccharide. Alternatively the precursors of the nucleotide sugars may not be available in old cells.

Early stationary and log phase cells showed equal ability to synthesize polysaccharide in washed cell suspensions and equal levels of the enzymes phosphoglucose isomerase, phosphomannose isomerase and UDPG dehydrogenase were found in both. Hexokinase, UDPG pyrophosphorylase and GDPM pyrophosphorylase levels dropped in the early stationary phase cells compared to log phase cells. However it seemed unlikely that this small drop would create a rate-limiting step in the synthesis of the extracellular slime.

Analyses were done to demonstrate whether any variation occurred during growth in the levels of the sugars present in the soluble pool of the cells. AlS1 cells were grown in yeast extract medium at 34° and harvested after 1, 3, 6, 24, and 48 hours incubation. They were extracted with 70% ethanol for 10 minutes at 70°. The cell debris was deposited by centrifugation and the supernatant concentrated to a small volume under reduced pressure. The absorbance at 260mμ was found giving a measure of the nucleotide concentration. Protein, phosphorus, total carbohydrate and uronic acid levels were estimated and glucose and galactose after hydrolysis with 0.01N HCl for 10 minutes at 100°. It was assumed that the nucleotide concentration would be approximately the same per cell at all stages of growth and the concentration of the other constituents were related to it. The results are shown in Fig.22 which gives the levels of these components /E260 at each time.

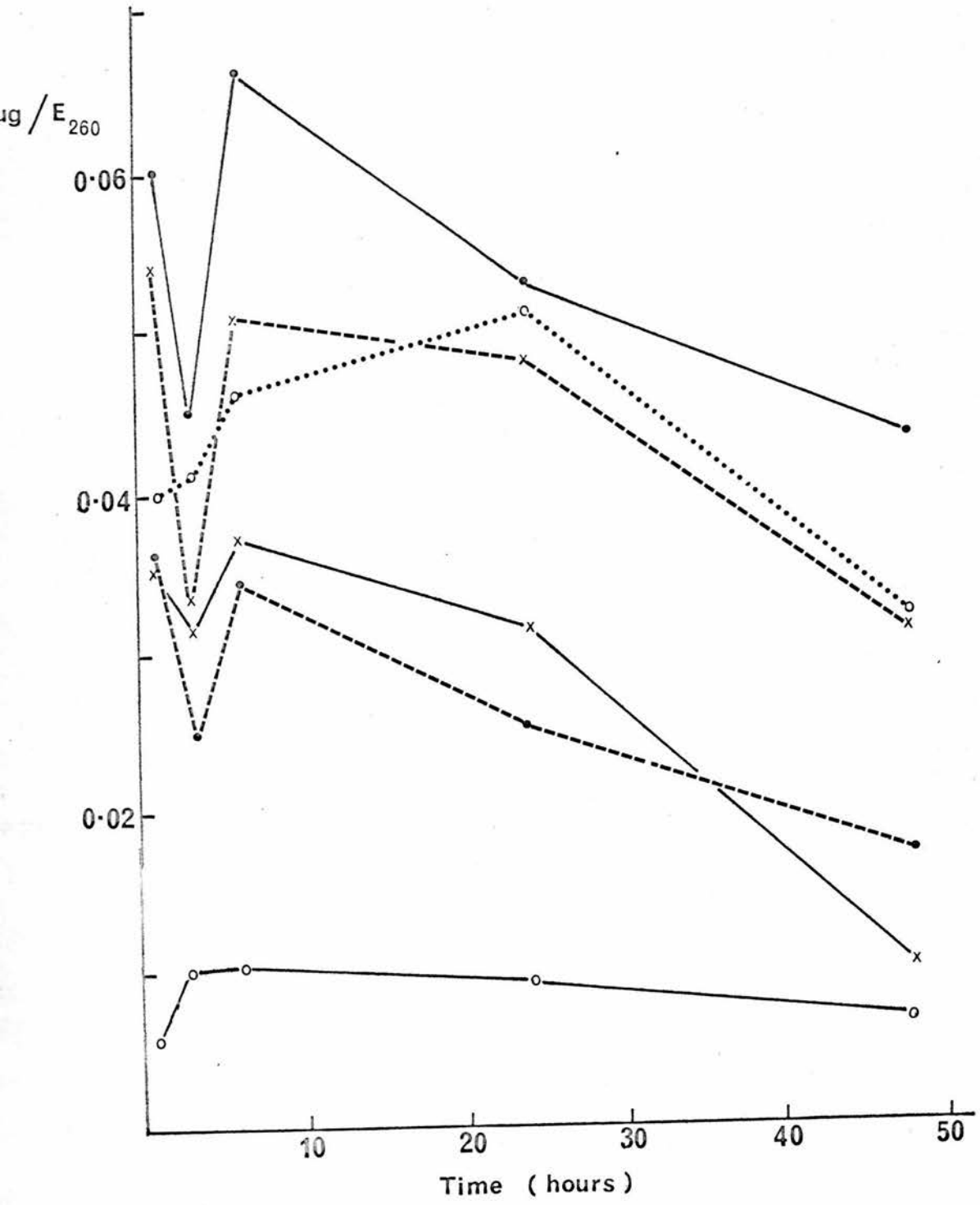


FIG 22.

Amounts of Carbohydrate (•——•), Phosphorus (o···o), Uronic acid (x--x), Glucose (•---•), Galactose (x——x) and Protein (o——o) in the Soluble Pool during Growth.

The levels of carbohydrate, glucose, galactose and uronic acid were high initially but dropped after 3 hours incubation. A rise after 6 hours incubation followed and these levels gradually fell as the culture aged. The concentration of phosphorus rose for the first 24 hours, then fell again. The protein concentration fell slowly throughout the incubation time after the first 3 hours when the level in the cell almost doubled.

Ethanol will extract all the soluble intermediates in the pool, such as nucleotides, nucleotide sugars, intermediates of sugar metabolism, cofactors such as FAD, NADP, and so on. The high level of sugars after incubation of one hour was probably due to uptake of glucose by the cells after inoculation into fresh medium and its conversion to other sugar derivatives. The culture will enter log phase after a period of adaptation and the levels of these sugars may fall as they provide structural units for cell wall synthesis and energy for cell growth and division. As the culture enters stationary phase, the intermediates of sugar metabolism will not be required for synthesis of cell wall polymers. More may be available for the formation of extracellular polysaccharide. This may be reflected by a rise in concentration of the nucleotide sugars. As the nutrients of the medium were exhausted, so the levels of sugars, phosphorus and protein in the soluble pool fell.

In early stationary phase cells and log phase cells, the soluble pool thus seemed to contain about equal quantities of the sugars required to synthesize polysaccharides, although it was not determined how much of these were in the form of the particular nucleotide sugars. 48 hour old cells, on the other hand, possessed lower levels of these sugars, phosphorus and protein which may account, in part at least, for their poor synthetic ability in washed cell suspensions.

c) ENZYME LEVELS OF CELLS GROWN IN VARIOUS MEDIA

It is known that cells grown in various media synthesize different quantities of exopolysaccharide depending on the composition of the medium. In particular the amount depends on the relative concentrations of carbon and nitrogen. Nutrient broth is a balanced medium in this respect, while minimal medium and yeast extract medium are carbon-rich and nitrogen-deficient. Whether variation in the levels of enzymes synthesizing the nucleotide sugar precursors of the exopolysaccharide occurs during growth in these different media was not known.

The enzyme levels of the cells of AlS1 were examined after growth in nutrient broth, minimal medium and yeast extract medium for 15 hours at 30^{o}. Exopolysaccharide present in the supernatant as slime was measured by the anthrone method and growth by the concentration of cellular protein. Polysaccharide production per mg cellular protein will then give an indication of the relative synthesis of polysaccharide in each medium. The specific activities of the enzymes from cells grown in each medium are shown in Table 8.

Polysaccharide production per mg cellular protein in yeast extract medium was about twice that in nutrient broth. In minimal medium it was midway between these two levels. However the specific activities of the enzymes showed hardly any change between cells grown in one medium and another, and certainly no large variation occurred. Whether any of the reactions assayed represented a rate-limiting step in the formation of polysaccharide was known. It is possible that polysaccharide synthesis may be controlled by the activities of other enzymes not able to be assayed, such as transferases or polymerases, or by the availability of intermediates other than the nucleotide sugars.

The levels of enzymes of AlS1 cells were also found after growth in

TABLE 8.

Enzyme levels of cells grown in various media

	Nutrient broth	Minimal Medium	Yeast extract Medium
µg polysaccharide/mg protein	16	20	27
hexokinase	11.2	16.2	11.2
Glc-6-P dehydrogenase	67	67	73
phosphoglucose isomerase	112	106	140
phosphomannose isomerase	19	19	16.8
UDPG pyrophosphorylase	19	16.8	16.8
GDPfucose synthetase	0.06	0.06	0.11
UDPG dehydrogenase	0.39	0.06	0.11

TABLE 9.

Enzyme levels of cells grown in various media

	I	II	III	IV
µg polysaccharide/mg protein	30	150	30	120
hexokinase	9.0	14	17.9	18.5
Glc-6-P dehydrogenase	201	140	179	168
phosphoglucose isomerase	245	174	224	174
phosphomannose isomerase	25.8	22.4	33.6	26.3
UDPG pyrophosphorylase	50.4	112	90	168
GDPM pyrophosphorylase	1.7	3.4	1.5	3.4
UDPG dehydrogenase	0.67	0.28	0.06	0.34
GDPfucose synthetase	0.06	0.17	0.06	0.17

various simple synthetic media. Again comparisons were made with the synthesis of polysaccharide in each medium and also with the abilities of washed cell suspensions prepared from these media to produce polysaccharide. The cells were grown at 32° for 15 hours in a basic medium containing 1% Na_2HPO_4, 0.3% KH_2PO_4, 0.1% NaCl, 0.1% K_2SO_4, 0.02% $MgSO_4$ with the following additions :

I) 1% $(NH_4)_2 SO_4$, 1% glucose

II) 0.03% $(NH_4)_2 SO_4$, 1% glucose

III) 2% casamino acids

IV) 0.03% $(NH_4)_2 SO_4$, 1% mannose

After incubation, the polysaccharide present in the supernatant per mg cellular protein was measured. The specific activities of the enzymes are shown in Table 9.

Medium II and IV were carbon-rich and nitrogen-deficient while I was more balanced and III tended to be nitrogen-rich. Polysaccharide produced by cells grown in II and IV was three to four times as much as that produced by cells grown in I and III. However the enzyme assays indicated little difference in specific activities of cells from the various media. The largest variation was in the levels of GDPM pyrophosphorylase and GDPfucose synthetase which had half the activity in cells grown in media I and III compared to II and IV. These two enzymes may be concerned solely with synthesis of GDPfucose for incorporation into extracellular polysaccharide. However the variations did not seem very significant particularly as these two assays were not very accurate. Thus, although polysaccharide production by the whole culture differed considerably in the various media, there was little alteration in the activity of the enzymes required to synthesize the nucleotide sugar precursors. In washed cell suspension experiments discussed previously, it was shown that cells from media I

TABLE 10.

ENZYME LEVELS OF CELLS GROWN AT DIFFERENT INCUBATION TEMPERATURES.

	22°	37°
µg polysaccharide/mg protein	138	64
hexokinase	14.5	22.4
glc-6-P dehydrogenase	44	73
UDPG pyrophosphorylase	49	50
GDPM pyrophosphorylase	3.4	2.3
GDPfucose synthetase	0.06	0.22
UDPG dehydrogenase	0.22	0.33

and II had equal ability to synthesize extracellular polysaccharide while cells from medium III produced polysaccharide at half this rate. This indicated the inherent ability of the cells, when placed in a suitable medium, to synthesize polysaccharide and the probability that the enzymes were already present to allow synthesis of the precursors.

Unlike A1S1 where mannose-6-P is required for synthesis of fucose, a constituent of the polysaccharide, the exopolysaccharide of A4 does not contain fucose or mannose. Cells of A4 were therefore grown in media II and IV to ascertain if the level of phosphomannose isomerase was altered by the presence or absence of mannose in the growth medium. The specific activity of the enzyme was found to be identical in cells from both media. This enzyme therefore may be constitutive in both A1S1 and A4 strains.

d) ENZYME LEVELS OF CELLS GROWN AT VARIOUS INCUBATION TEMPERATURES

It was known that growth at different incubation temperatures induced production of varying amounts of exopolysaccharide. Generally the lower the incubation temperature the more was produced. A comparison was therefore made between polysaccharide synthesis and the specific activities of enzymes of A1S1 cells grown at two incubation temperatures.

The cultures were grown in minimal medium at 22° and 37° for 24 hours. Polysaccharide production /mg cellular protein was measured at the end of this time and the enzymes in the cells assayed. The results are shown in Table 10.

After 24 hours incubation, the cells grown at 22° had synthesized twice as much polysaccharide as cells at 37°. However the enzyme assays did not reflect this difference. The specific activities did not change very much at the two incubation temperatures and any variation found did not seem to be

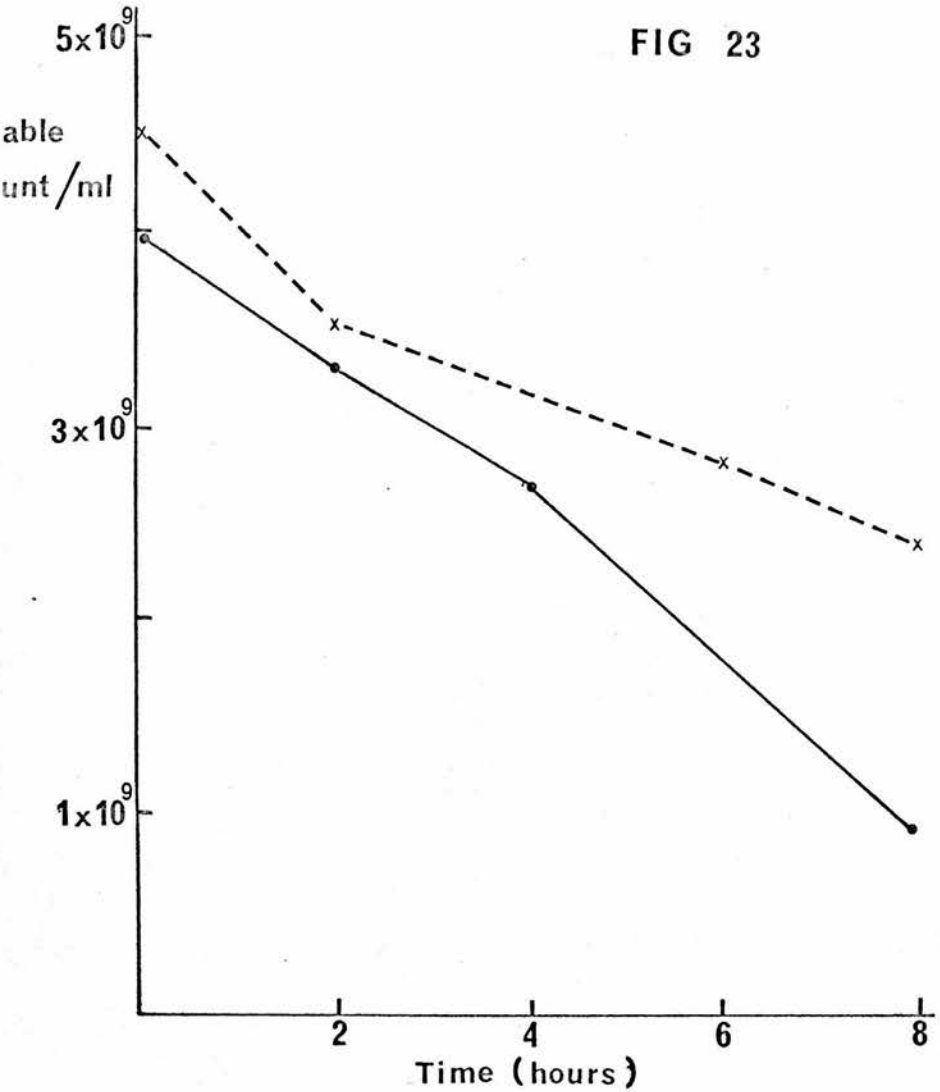
sufficient to account for the difference in quantity of polysaccharide produced. The difference may be accounted for merely by the change in growth rate at the two temperatures. At 37° most of the metabolic activity of the cells may be engaged in growth and cell division, but at 22°, as these processes do not occur so rapidly, more energy and metabolites may be available for synthesis of exopolysaccharide. However greater synthesis of exopolysaccharide was not accompanied by a rise in the specific activity of the enzymes synthesizing the nucleotide sugar precursors of the polysaccharide.

POSSIBLE FUNCTIONS OF THE CAPSULE

A role has not been definitely assigned to the capsule or slime of saprophytic organisms. This material has, amongst other things, been suggested to act as a protective coating for the cell against dessication (Morgan and Beckwith, 1939) or attack by bacteriophage (Kauffmann and Vahlne, 1945). Due to the negative charge conferred by the capsule it may also aid in the uptake of ions (Rorem, 1955), in addition to the dispersal of the cells.

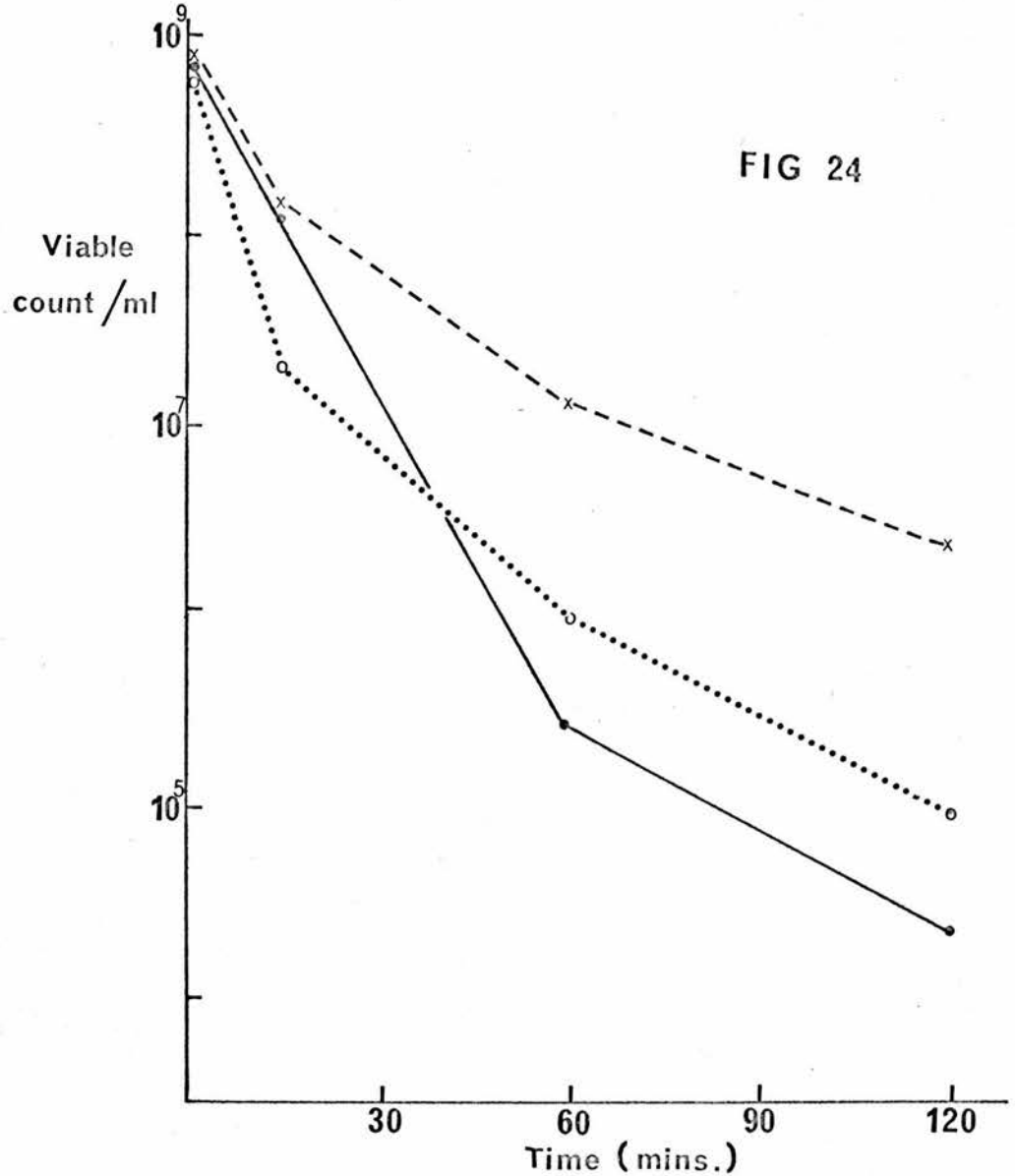
Ca^{++} and other divalent ions are known to function as salt bridges binding monosaccharides on the surface of the cell wall (Humphrey and Vincent, 1962). The action of EDTA, a chelating agent, is to remove these inorganic cations causing loss of lipid, lipoprotein or lipopolysaccharide from the cell wall (Gray and Wilkinson, 1965). It is also known that loss or decrease of somatic antigens at the cell surface decreases the resistance of Gram-negative organisms to deleterious agents such as lysozyme (Herzberg and Green, 1964). Organic cations acting with EDTA break salt bridges forming bonds with anionic polymers such as lipoprotein and lipopolysaccharide on the cell surface, these making the organism more susceptible to lysozyme (Voss, 1967). It was thought that

FIG 23



Viable count /ml during incubation of AI (x--x) and AI(O) cells (o—o) with EDTA

FIG 24



Viable count /ml during incubation of AI (x--x), AI(O) (o—o) and AISI cells (o···o) with EDTA-Tris-lysozyme

perhaps the presence of a capsule may protect the cell surface from attack of this kind. Thus the effect of EDTA , in the first place, on the viability of the saline suspension of A1 cells and of a non-capsulated mutant of A1, A1(0), was found. Secondly, the effect of EDTA, Tris and lysozyme together was demonstrated. Tris has been shown to act as an inorganic cation (Voss, 1967). Finally, any differences in the survival of A1 and A1(0) cells during drying , and during attack by phage were found.

a) Effect of EDTA.

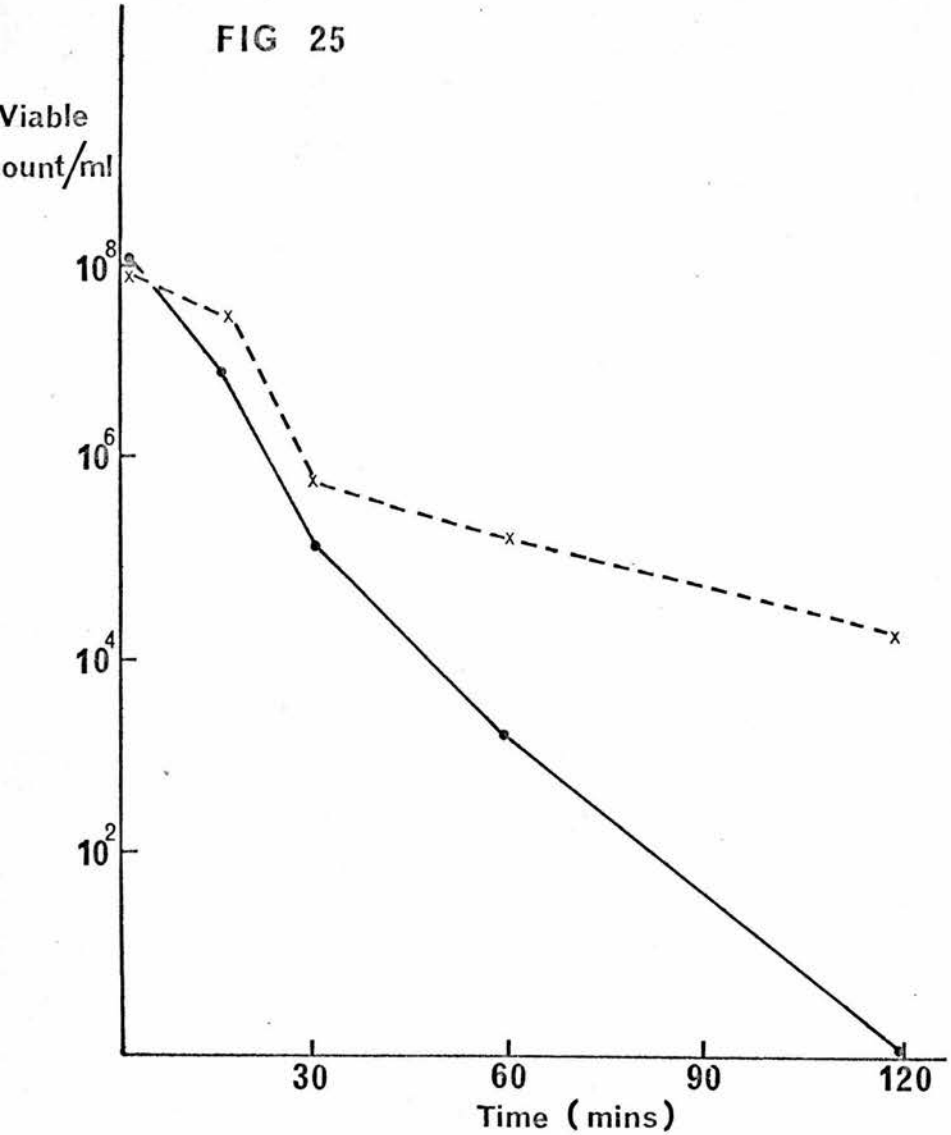
Cells of A1 and A1(0) were grown in minimal medium for 15 hours at 34°. They were harvested by centrifugation and washed in saline. To 10 mls cell suspension was added 6 mgs EDTA-disodium salt and the cultures were incubated at 37°. At intervals viable counts were made by the method of Miles and Misra (1938). Results are shown in Fig.23.

The viable count of the A1 culture dropped by almost half over the incubation period, that for A1(0) dropped by four-fold. Thus a capsulate organism survives this treatment better than a non-capsulate strain. The capsule may prevent the ions on the cell surface from chelating with EDTA and so enhance survival. However the capsule itself may also contain ions which will chelate with EDTA, and protection of the cell surface may occur indirectly in this way.

b) Effect of EDTA, Tris and lysozyme.

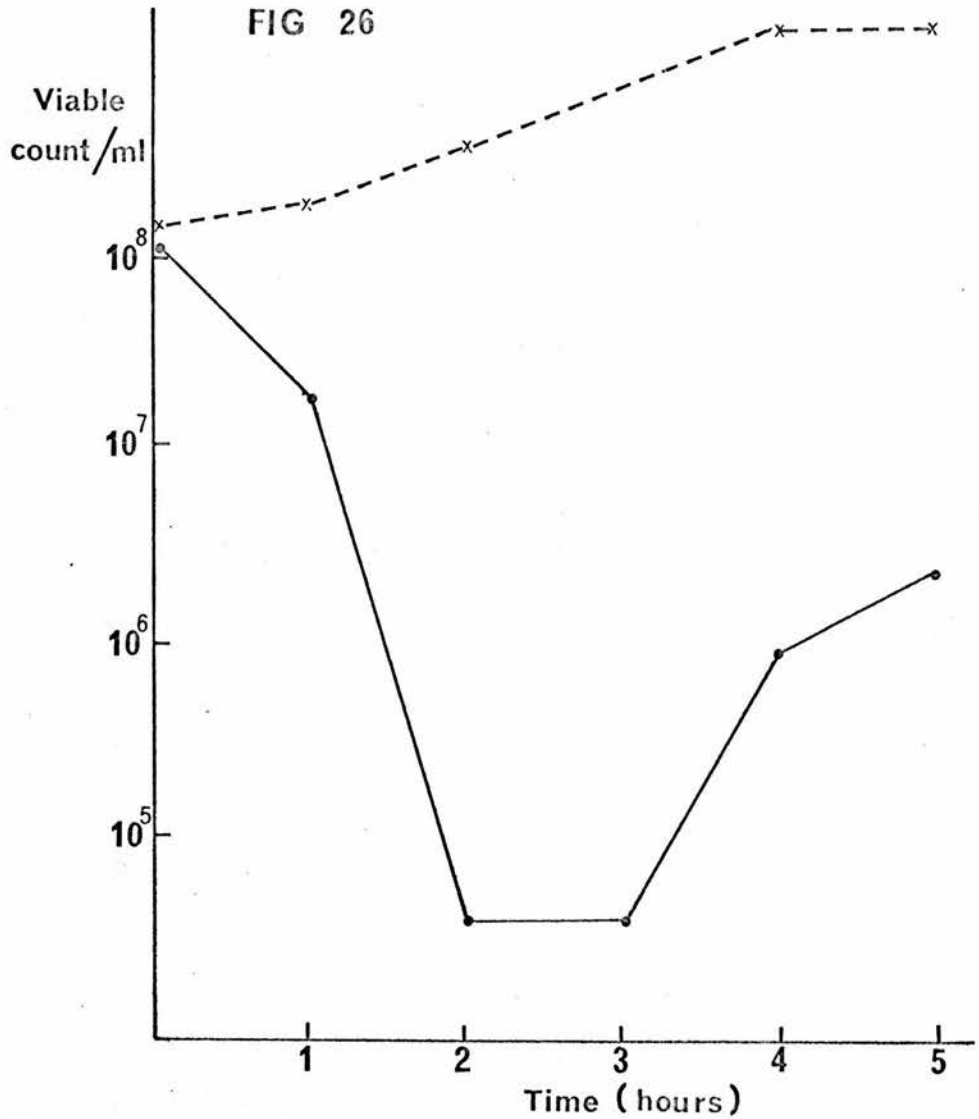
Cells of A1, A1S1 and A1(0) were grown in minimal medium for 15 hours at 34°. The cells were harvested by centrifugation and washed in saline. To 10 mls cell suspension was added 2 mgs EDTA-disodium salt, 92 mgs Tris and 0.33 mgs lysozyme. The cultures were incubated at 35° and samples were

FIG 25



Effect of drying on the viability of AI (x--x) and AI(O) (•—•) cells

FIG 26



Effect of phage on the viability of AI (x--x) and AI(O) (•—•) cells

removed at intervals, viable counts being made by the method of Miles and Misra (1938). The results are given in Fig. 24.

The protection offered by the capsule against attack by an organic cation and lysozyme was quite distinct. At the end of the 2 hours incubation period, one hundred times more cells of A1 survived compared to A1(0). The protection offered by extracellular polysaccharide in the form of slime was slight compared to that of the capsule.

c) Effect of drying.

Cells of A1 and A1(0) were grown in minimal medium at 34° for 15 hours. The cells were harvested by centrifugation and washed in saline. Previously 1½" squares of Whatman No. 1 paper had been put inside petri plates and sterilized. One drop of each culture from a standard pipette was placed on each square of paper. The plates were incubated at 30° without lids. At intervals one piece of paper was removed and placed in a tube containing sterile saline. The tube was shaken vigorously and viable counts performed on this suspension by the method of Miles and Misra. A graph of the results obtained is shown in Fig. 25.

A marked difference emerged from the survival curves of the capsulated and non-capsulated bacteria. No A1(0) cells survived by the drying procedure for 120 minutes, whereas about half the original number of A1 cells survived 120 minutes or more. This may be of value in the natural environment where drought and dessication may occur frequently.

d) Effect of phage

To log phase cultures of A1 and A1(0) growing in 100 mls minimal medium

was added 1ml A1 phage preparation (approx. 3×10^6 plaque forming units/ml). Incubation was continued at 34°. At intervals samples were removed and viable counts determined by the method of Miles and Misra. The results are given in Fig. 26.

A clear distinction emerged from graphs of the viable counts of the capsulate and non-capsulate strains. No drop in viable count of A1 cells occurred although the culture did not continue to grow exponentially. There was a drop in the viable counts of the A1(0) culture from an original level of 10^8 cells/ml to 6×10^4/ml before cell division started again. The capsule therefore seemed to protect the cells to a considerable extent against attack by phage when comparison was made with cells of a non-capsulate mutant.

It may be concluded then from this set of experiments that the capsule confers on K. aerogenes A1 protection against dessication, chelating agents and attack by phage or by organic cations and lysozyme. None of these conditions are liable to occur in the laboratory and whether any ever occur in the natural environment is not known. Drought and dessication seem probable however, and chelating agents and cations may occur in the soil, together with the specific phages.

RESULTS

SECTION B

NON-CAPSULATE MUTANTS

Mutants of K. aerogenes A4, A1 and A1S1, which are unable to synthesize extracellular polysaccharide material, were found to occur spontaneously and after treatment of the culture with various mutagens. The viability of the cell was not altered. The mutants were easily distinguished from their parents by their colonial appearance after growth on media such as EMB glucose, yeast extract or minimal medium for several days at 30°. Plates 3 and 4 show the cultural characteristics of non-capsulate mutants of A4 and A1.

After mutagenesis, the cultures were generally grown in nutrient broth for several generations to allow phenotypic expression of the mutation. 0.1ml of appropriate dilutions of the culture in saline were then spread over the surface of the plates. Each plate, after incubation, was examined for non-mucoid colonies. A procedure to select non-mucoid cells from a culture containing both mucoid and non-mucoid types was not found. It was thought that the smaller size of the non-capsulate organism may allow it to be separated from the capsulate organism in the culture using a method such as sucrose gradient centrifugation. A gradient from 40 - 10% sucrose was tried. The culture was layered on top and centrifugation was carried out at 5,000g for 30 minutes using a swing-out head. Non-capsulate cells tended to be found in the lower layers although they also occurred in other layers. It was difficult to set up such a gradient under sterile conditions and as separation of capsulate and non-capsulate cells was not by any means absolute, the technique was abandoned.

Spontaneous mutation to strains not synthesizing a capsule or slime occurred at low frequency, the rate being increased by the use of mutagens. For example

TABLE 11.

Reversion of A4(0) to A4

Mutagen	Number of revertants	Total number of colonies.
None (grown in NB at 37° for 72 hrs.)	0	2,000
45°	0	1,200
$MnCl_2$	0	2,000
MNG	0	2,000
uv	0	600
Total	0	7,800

TABLE 12.

Reversion of A1(0) to A1

Mutagen	Number of revertants	Total number of colonies
uv for 4-8 mins.	0	200
ɣ-rays for 4-8 mins.	0	400
MNG	0	300
$MnCl_2$	0	500
Total	0	1,400

in A4, mutation to A4(0) occurred spontaneously at a rate of 0.08%; use of the mutagen 2-aminopurine increased the rate to 0.3%. One mutagen did not appear to be more efficient than another in inducing this mutation. A variety of mutagens was used in an attempt to induce mutation at different loci on the chromosome.

REVERSION

It was noted during the course of this work that spontaneous reversion of these non-capsulate mutants to polysaccharide-producing strains never seemed to occur. The mutation appeared to be irreversible. Many methods employing various mutagens were used to try to induce this reversion. Some examples are shown in Table II, where a non-mucoid mutant of A4 obtained by use of HNO_2 was treated with several mutagens. Various mutagens were also used during growth of non-mucoid mutants of A1S1 which had been obtained by use of aminopurine, acriflavine and Y-rays, and non-mucoid mutants of A1 obtained by use of Y-rays and acriflavine. An example of the procedures tried with an A1(0) mutant is shown in Table 12. No revertants were ever found.

As no success had been achieved using these methods, it was wondered whether a primer such as an oligosaccharide component of the polysaccharide may need to be present inside the cells before synthesis of the polysaccharide would start. Plating out after mutagen treatment was therefore tried on yeast extract medium containing the appropriate polysaccharide, either autoclaved which will permit slight breakdown of the macromolecular structure, or partially hydrolysed in 0.1N H_2SO_4 for 30 minutes at 100° prior to autoclaving. Non-mucoid mutants of A1, A1S1 and A4 were treated with mutagens such as aminopurine and HNO_2. Altogether over 6,000 colonies were examined and no revertants were found.

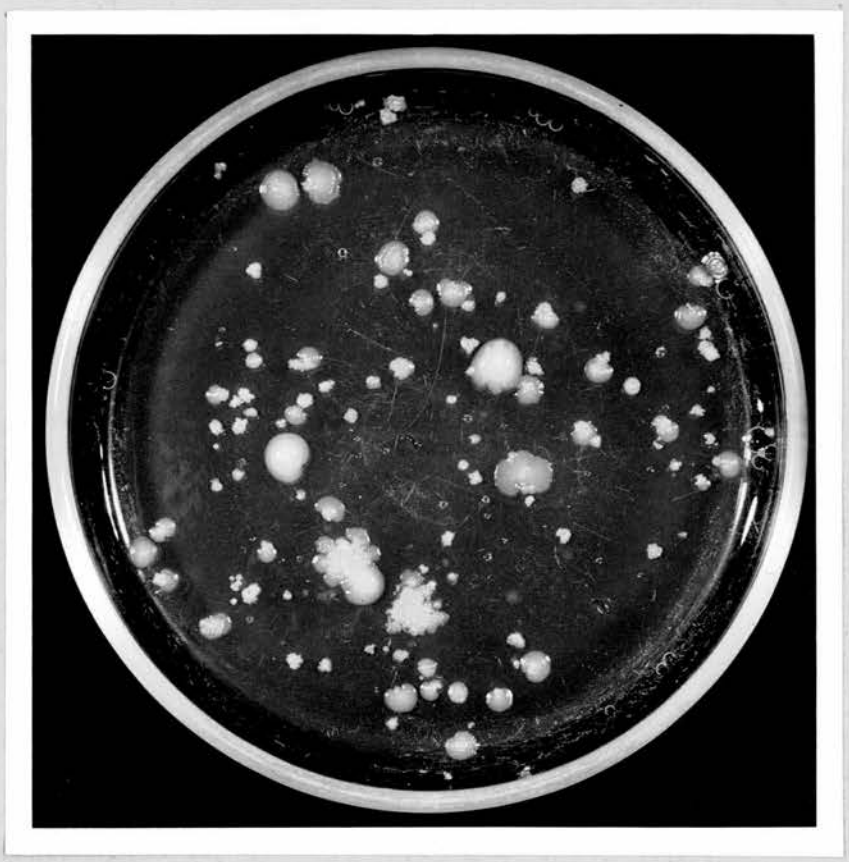


Plate 6. *K. aerogenes* A1(0) after growth on yeast extract medium at 33° for 96 hours. 3 mucoid colonies may be seen.

A cell-free extract of a strain of K. aerogenes, which lacks the enzyme UDPGal-4-epimerase and thus has no primer or preformed polysaccharide, incorporated galactose from UDPGal into the lipid fraction or all three of the component sugars from their appropriate nucleotide sugars into polysaccharide material (Troy and Heath, 1968). In this case, a primer was not required before synthesis of the polysaccharide occurred. However it has been shown that in cell-free extracts of Pneumococcus Type III a primer of 8-12 repeating units is required for synthesis of the polysaccharide (Mills and Smith, 1965). A primer is also thought to be required before synthesis of hyaluronic acid occurs (Markovitz, Cifonelli and Dorfman, 1959). The failure to obtain revertants may be due to the primer being of incorrect length and structure or being unable to penetrate the cell. However it seems more likely that there may be other reasons unassociated with an acceptor molecule.

If reversion even in the presence of a mutagen were to occur at very low frequency - say 1 in 10^7 cells, then it may be that not sufficient colonies have been examined to find a revertant. A selective technique was required. It has been suggested that one of the functions of a capsule may be to protect the cell against attack by phage. It has already been shown that the capsule of A1 protects the bacterium to a considerable extent from attack by an A1 phage. So growth of several A1(0) mutants after mutagenesis in the presence of the A1 phage was tried in an effort to obtain some selection of mucoid revertant cells, if any occurred.

Several non-capsulate mutants of A1 were treated with the mutagens MNG, acriflavine and EMS. After mutagenesis the cultures were grown until they were in log phase, A1 phage was added and incubation continued for a further 3 hours.

TABLE 13.

Mucoid revertants after growth with A1 phage.

Strain of A1(0)	Mutagen used	Number of mucoid colonies	Total number of colonies
01 ($MnCl_2$)	MNG	1	1,000
02 (AP)	MNG	1	200
	EMS	0	200
03 (MNG)	acrif	3	200
	MNG	1	200
	EMS	0	200
04 (acrif)	EMS	2	200
	MNG	5	200
05 (acrif)	MNG	1	1,000
06 (HNO_2)	MNG	0	200
07 (45°)	EMS	0	600
08 (caffeine)	MNG	0	200
09 (γ-rays)	EMS	0	200

Dilutions were spread on yeast extract medium. The plates were examined after several days incubation at 33° to find any mucoid colonies. Plate 6 shows the typical appearance of the colonies. Table 13 gives the results obtained.

It may be seen that in about half the strains of A1(0) used, a few mucoid colonies were obtained as a result of growth with the A1 phage. The frequency of their appearance is fairly high and is perhaps greater than would be expected even if the phage were acting as a partial selective agent. The possibility was considered that the phage particles may themselves be carrying sufficient genetic information to enable recombination and repair to take place within the non-mucoid mutant. The mucoid colonies then would be the result of low frequency transduction and not of reversion.

This hypothesis may be tested in three ways. If the phage genome is present within the cell, then the cell will be immune to attack by the same phage. Secondly, induction of the phage by uv light may occur, and finally phage prepared by growth on A1(0) cells as host instead of A1 may not contain the genes required to repair the deletions in non-capsulate strains.

The mucoid strains were seeded on yeast extract plates and tested with drops of the A1 phage preparation. The parent strain A1 and A1(0) strains were tested similarily. In addition the phage was added to log phase cultures in nutrient broth and the change in turbidity over the next 3 hours followed by measuring the absorbance at 540mμ. The mucoid strains obtained by growth with phage were resistant to the phage. The parent A1 and the non-mucoid strains were susceptible.

Each of the mucoid strains was grown in nutrient broth overnight at 37°. The cells were harvested, washed with saline and induced with uv light for a period of 30 seconds. The cells were deposited by centrifugation. One drop of the

supernatant was used to spot on freshly prepared lawns of A1 and an A1(0) strain. Incubation was carried out for 4 hours and overnight at 30°. Plaque formation was recorded as shown in Table 14.

It may be seen that induction of phage occurred with the mucoid strains of 04 but not with the other strains. These were re-tested using varying times of exposure to uv light but induction of virulent phage was not obtained.

The A1 phage was regrown with A1 and an A1(0) strain as host using the method described by Sutherland and Wilkinson (1965). The non-mucoid strains 01 - 05 were grown in the presence of the A1 and A1(0) phage after mutagenesis. The results are shown in Table 15.

In most cases mucoid colonies were found whether the phage had been prepared by growth in A1 or A1(0) culture. It seems then that if low frequency transduction is occurring, the phage genome has retained sufficient genetic information to enable the exopolysaccharide to be synthesized even after growth of the phage on an A1(0) host. Phage-mediated transduction has often been reported in Salmonella, Escherichia, Shigella and Proteus species but until recently it had not been found in K. aerogenes. MacPhee, Sutherland and Wilkinson (1969) described a phage of K. aerogenes W70 which would participate in generalised transduction. The markers involved in genetic exchange included arginine, lysine, threonine, leucine, isoleucine, valine, proline, cysteine, adenine, uracil, maltose utilization and streptomycin resistance but mucoidness was not transduced. It seems possible that phage-mediated transduction of mucoidness may be occurring here, especially as no A1(0) mutant was found to revert spontaneously or on mutagenesis to the capsulate form.

The mucoid revertants had the same biochemical reactions as A1 and cannot

TABLE 16.

Rate of mutation of non-mucoid strains

	Spontaneous	Acriflavine
A1	0.19%	0.6%
Mucoid revertant of 02	0.09%	0.4%
" " " 03	0.12%	0.45%

TABLE 17.

Rate of mutation to non-mucoid strains

	Spontaneous	Aminopurine	$CoCl_2$	Acriflavine
A1	0	0.3%	0.4%	0.4%
A1S1	0.08%	0.6%	0.7%	0.5%

be distinguished from A1 in wet India ink films. The capsular polysaccharides of two of them were prepared and compared with that of A1. All three polysaccharides contained the same component sugars glucuronic acid, glucose and fucose and no other sugars. The % glucose was the same indicating that probably the sugars were present in the same proportions as in A1.

The ability of the revertants to retain the capsule was compared to that of A1. Results were obtained for the rate of spontaneous mutation to non-mucoid colonies and using acriflavine. These are shown in Table 16. The stability of the revertants was found to be approximately the same as A1 both in spontaneous loss of ability to produce a capsule and when acriflavine was used as a mutagen.

In two cases it has been shown that the ability to produce a capsule is carried episomally (Ørskov and Ørskov, 1966; Hardy and Nell, 1967). However, in the first the capsule was unusual in that it was protein in nature and not carbohydrate. The polysaccharide produced in the second case was thought to be colanic acid. It was decided to test whether the genes determining or regulating production of the capsule in A1, A1S1 and A4 might be carried episomally. This would explain why reversion from a non-capsulate strain never occurred. If an episome is involved then curing of the culture using acriflavine or $CoCl_2$ should be possible. Also the episome should be transferable to suitable recipient cells inducing the production of polysaccharide.

The first was tested by growing cultures of A1 and A1S1 in nutrient broth and in nutrient broth containing 0.2M $CoCl_2$, acriflavine (100µg/ml) or 2-aminopurine (200µg/ml) for 48 hours at 37°. Dilutions of the cultures were spread on yeast extract plates and after incubation the number of non-mucoid colonies counted. The results are given in Table 17.

It may be seen from these figures that, although $CoCl_2$ and acriflavine increased the number of non-mucoid mutants from those arising spontaneously, the increase was not significantly greater than by use of aminopurine. Curing therefore was not shown to take place.

To ascertain if transmission of capulation occurred to a non-capsulate recipient, histidine-requiring mutants of the parent strains A4, A1 and A1S1 were obtained as donors. The auxotrophs were isolated by use of penicillin selection after treatment with aminopurine, acriflavine, γ-rays or uv light. Pairs of auxotrophic, polysaccharide-synthesizing donors and non-capsulate recipients were grown together in nutrient broth overnight at 37° and 0.1 ml used to inoculate a fresh tube of nutrient broth which was incubated again overnight. Dilutions were spread over minimal medium. No capsulate colonies were found after incubation for several days at 30°. It was concluded that no transmission of capulation had taken place under the conditions of the experiment.

It has been shown that in certain non-mucoid strains of Salmonella, p-fluorophenylalanine has the effect of derepressing formation of the exopolysaccharide, colanic acid (Kang and Markovitz, 1966; Grant, 1968). Strains thought previously to be non-mucoid will synthesize colanic acid in the presence of pFA. Several non-mucoid mutants of A1, A1S1 and A4 were grown with pFA and plated out on minimal medium containing pFA. No mucoid colonies were produced even on prolonged incubation, such as 7 days at 32°. Production of slime or a capsule in these strains of K. aerogenes therefore does not appear to be under the same control mechanism and regulation as the colanic acid system. Furthermore pFA was shown to have no effect on the quantity of polysaccharide synthesized by A1S1.

TABLE 18.

Mutagens used to obtain non-mucoid mutants

Parent strain	A1	A1S1	A4
	HNO_2	acriflavine	uv light
	acriflavine	EMS	45° incubation
	"old" culture	$MnCl_2$	acriflavine
	$MnCl_2$	"old" culture	$MnCl_2$
	aminopurine	aminopurine	HNO_2
	45° incubation	ɣ-rays	"old" culture
	caffeine	MNG	
	ɣ-rays		aminopurine
	MNG		

It was concluded that mutation of capsulate and slime-producing strains of K. aerogenes to non-mucoid strains occurred at low frequency. The rate was increased by the use of various mutagens. Reversion back to a polysaccharide-producing strain never occurred spontaneously or after mutagenesis. A primer was not shown to be required for reversion and the only method of regaining the polysaccharide-synthesizing ability was to grow the non-mucoid strains in the presence of a phage. Low frequency transduction may have occurred. The revertants were immune to the phage and in one case could be induced by uv light to produce virulent phage. The ability to produce the capsule was not shown to be carried episomally. Curing by acriflavine or $CoCl_2$ did not occur nor transmission of capsulation. pFA did not derepress polysaccharide synthesis in non-capsulate strains.

ENZYME LEVELS OF NON-CAPSULATE STRAINS

Non-capsulate mutants of A4, A1 and A1S1 were obtained using most of the mutagens outlined in the Methods. The parent strains and the mutagens used are listed in Table 18. Each strain was grown in minimal medium at 34° for 15 hours. In the case of the A1 and A1S1 mutants the enzymes hexokinase, Glc-6-P dehydrogenase, phosphoglucose isomerase, phosphomannose isomerase, UDPG pyrophosphorylase, GDPM pyrophosphorylase, GDPfucose synthetase and UDPG dehydrogenase were assayed. In the case of the A4 mutants hexokinase, glc-6-P dehydrogenase, phosphoglucose isomerase, phosphomannose isomerase, UDPG pyrophosphorylase, UDPGal-4-epimerase and UDPG dehydrogenase were assayed. The specific activities are given in Table 19. This represents an average of all the non-capsulate mutants of the particular strain listed in Table 18.

The mutants possessed all the enzymes assayed, and, except in a few instances

TABLE 19.

Enzyme levels of non-capsulate mutants

	A1(0)	A1S1(0)	A4(0)
Number of strains used	17	9	13
Hexokinase	45	40	33.6
Glc-6-P dehydrogenase	67	56	21.3
Phosphoglucose isomerase	78	51	78
Phosphomannose isomerase	20.7	12.3	21.3
UDPG pyrophosphorylase	35.3	45	39
GDPM pyrophosphorylase	3.4	3.4	-
GDPfucose synthetase	0.17	0.11	-
UDPG dehydrogenase	0.62	0.22	0.26
UDPGal-4-epimerase	-	-	8.4

TABLE 20.

Mutants with lowered enzyme levels

Parent strain	Mutagen	Enzyme altered	Specific activity of enzyme
A4	aminopurine	UDPG pyrophosphorylase	13
A1	caffeine	GDPfucose synthetase	0.05
A1	HNO_2	GDPM pyrophosphorylase	1
A1	acriflavine	UDPG pyrophosphorylase	12

where the level of a particular enzyme was lowered, the specific activities were similar to those of the mucoid parent strains. The mutants, where the level of an enzyme was lower than the average, are listed in Table 20. Therefore in all the non-mucoid strains tested, the enzymes were present to enable the synthesis of the nucleotide sugars required as intermediates in the synthesis of the exopolysaccharide. Mutation preventing this synthesis may have occurred at a later stage, such as in the transferase enzymes which are required for transfer of sugars from the nucleotide sugars to the polysaccharide or to an intermediate compound. If regulator genes are present like those described in the colanic acid system (Markovitz, 1964), mutation of these may stop synthesis of the polysaccharide. The genes controlling production of the enzymes synthesizing the nucleotide sugar precursors of the exopolysaccharides may be much less likely to mutate than these other genes. This is found in the majority of the rfb mutants of Salmonella which are able to synthesize the core of the lipopolysaccharide and the nucleotide sugars for the O-side chain, but are unable to add these sugars to the core (Nikaido, 1968). However it still seems unusual that, after the use of such a variety of mutagenic techniques, none of the enzymes involved in the synthesis of the nucleotide sugar precursors of the polysaccharide was found to be absent.

NUCLEOTIDE CONTENT OF AlS1(0) MUTANTS

The nucleotide sugar content of three non-mucoid mutants of AlS1 obtained by mutagenesis with aminopurine, ɣ-rays and EMS were compared to that of AlS1. These mutants had already been shown to possess all the enzymes enabling synthesis of the nucleotide sugars UDPG, GDPfucose and UDPGlUA. If a block in the

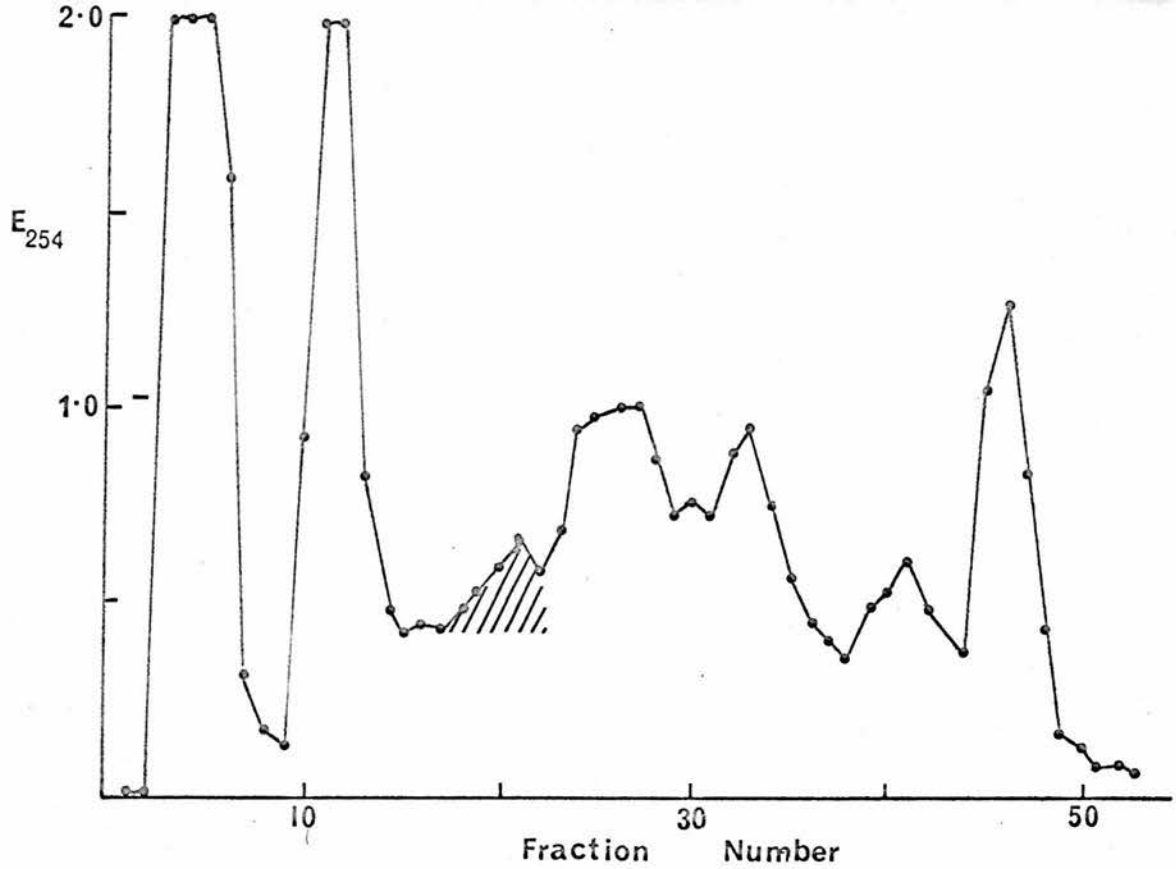


FIG 27. A1SI ; absorbance at 254mμ during elution

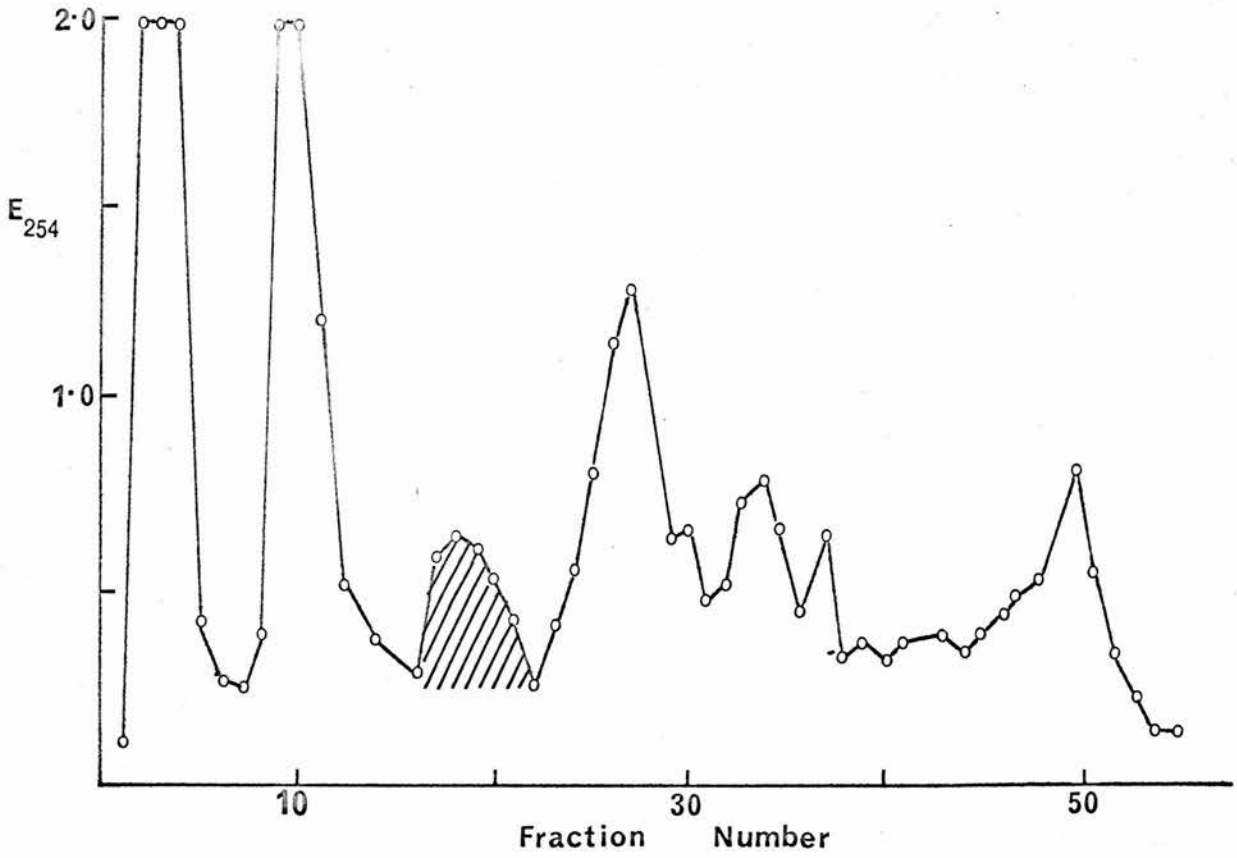


FIG 28. A1SI(O) ; absorbance at 254 mμ during elution

synthesis of the extracellular polysaccharide occurs at a later stage, then accumulation of one or more of the nucleotide sugars may occur.

Cultures of A1S1 and the three non-mucoid mutants were grown to log phase in nutrient broth at 33°. The cells were harvested and an ethanol extract prepared and purified by passing through a column of Ecteola cellulose. The absorbance at 254mμ of each fraction was measured as elution from the column occurred. Figs. 27 and 28 show the absorbance curves for A1S1 and one non-slime-producing mutant. The other two mutants gave similar results to these.

The general outline of the eluates of A1S1 and the non-mucoid mutants were the same. The results obtained by Grant (1968) showed the order of elution of the absorbing material. Peak III (shaded in Figs. 27 and 28) contained the majority of the nucleotide sugars, except for UDPGlUA which was not eluted until later, around Fraction Number 40. The tubes containing Peak III material were pooled, freeze dried and redissolved in a small quantity of water. Hydrolysis was carried out in 0.01N HCl at 100° for 10 minutes and analysis of glucose, galactose, fucose and total carbohydrate carried out. The results are shown in Table 21, the levels of the sugars being given as $\mu g/E_{254}$.

The total level of carbohydrate as measured by anthrone was higher in each of the three mutants than in A1S1. The level of glucose was higher in at least two of the three mutants, of galactose in two mutants and of fucose in all three. However these values were not greater than twice that of A1S1. The parent is able to utilize the nucleotide sugars UDPG and GDPfucose to synthesize extracellular polysaccharide. UDPG and UDPGal are required for synthesis of cell wall lipopolysaccharide and ADPG for glycogen synthesis. Fucose is the only nucleotide sugar estimated which is thought to be used solely in the exopolysaccharide production. Neither it nor the nucleotide sugars containing glucose

TABLE 21.

Levels of nucleotide sugars in AlS1 and 3 non-mucoid mutants

	AlS1	Mutant 1	Mutant 2	Mutant 3
$\mu g/E_{254}$				
carbohydrate	0.023	0.068	0.036	0.031
glucose	0.007	0.010	0.009	0.008
galactose	0.019	0.018	0.033	0.028
fucose	0.007	0.012	0.012	0.012

or galactose accumulated to a large extent in any of the three non-mucoid mutants. Total carbohydrate content of this peak was similarly not significantly higher than in AlSl. There may be feedback control of the synthesis of nucleotide sugars such as has been found in a number of other cases (Kornfeld and Ginsburg, 1966; Bernstein and Robbins, 1965) which would prevent all but a slight accumulation of the nucleotide sugars. Thus GDPfucose may inhibit the activity of the enzyme GDPMan hydro-lyase or GDPMan pyrophosphorylase depending whether GDPMan is required by the cell for synthesis of any other polysaccharide or nucleotide sugar, or whether it is merely an intermediate in the formation of GDPfucose. UDPG is unlikely under most circumstances to accumulate as it is an important intermediate in sugar nucleotide transformations such as in epimerization to UDPGal. It is also thought to be required in small quantities for the synthesis of lipopolysaccharide. Glucose is, in addition, required as the ADPG derivative for synthesis of glycogen, so that another control system may operate in the cell based on the relative availabilities of the nucleotide carriers.

ATTEMPTS TO OBTAIN ADDITIONAL MUTANTS DEFECTIVE IN SOME ASPECT OF EXTRACELLULAR POLYSACCHARIDE SYNTHESIS

a) The structure of the Salmonella lipopolysaccharide, core and O-side chains, has been determined largely by use of R mutants deficient in some aspect of sugar metabolism. These mutants may be defective in such enzymes as phosphoglucose isomerase (Fraenkel, et al, 1963), phosphomannose isomerase (Zeleznick, et al, 1965), UDPG pyrophosphorylase (Sundararajan, Rapin and Kalckar, 1962) or UDPGal-4-epimerase (Osborn, 1963). It was hoped to obtain mutants of K. aerogenes A1 and A4 similar to these, so that their extracellular polysaccharide production under various conditions and in cell-free systems may be studied. It has been noted

TABLE 22.

Levels of enzymes in A4 and a mutant of A4

	A4	Mutant grown on glucose	Mutant grown on galactose
Hexokinase	34	31	25
UDPGal-4-epimerase	8.4	4.5	5.0
UDPG pyrophosphorylase	39	0.2	4.5

already that all the non-capsulate mutants of A1, A4 and A1S1 found possessed the enzymes necessary for synthesis of the nucleotide sugar precursors of the polysaccharide. They also had the same sugar reactions as the parent strains.

The extracellular polysaccharide of A1 and A1S1 contains glucose, glucuronic acid and fucose. Penicillin selection techniques were used in conjunction with the usual mutagens to obtain mutants defective in the ability to utilize mannose or glucose as the sole source of carbon and energy. In an analogous manner the polysaccharide of A4 contains galactose, glucose and glucuronic acid and selective techniques were used to obtain mutants deficient in the ability to utilize galactose or glucose as the sole carbon and energy source. Treated cultures were plated out on minimal medium containing glucose, galactose or mannose, as appropriate. Alternatively the method of Nikaido (1966) employing EMBgal plates was used to obtain epimerase-less mutants.

Many attempts were made to find sugar-negative mutants. No success was achieved, except one mutant of A4 was recovered after treatment with EMS and penicillin selection which produced extracellular polysaccharide like the parent on minimal medium containing galactose but which produced distinctly less polysaccharide on minimal medium containing glucose. The mutant formed acid and gas from peptone water containing glucose only or galactose only after incubation at 37° for 24 hours. The amount of extracellular polysaccharide synthesized was found after growth of the culture in minimal medium containing glucose and containing galactose overnight at 33°. The cells were boiled to strip off the capsules.

	glucose-grown cells	galactose-grown cells
µg extracellular polysaccharide/mgm cellular protein	2	30

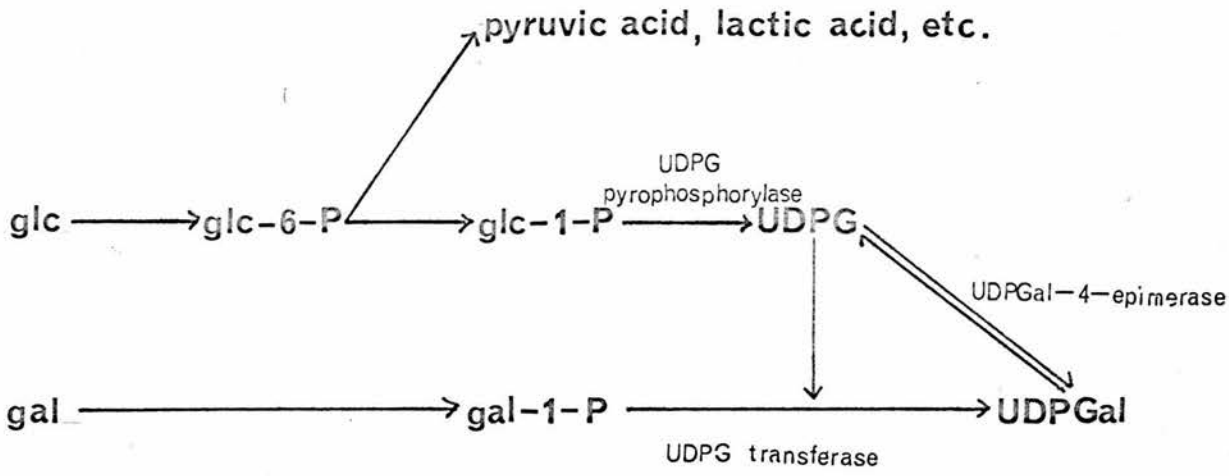


FIG 29. Intermediary metabolism in E.coli.

It was seen that extracellular polysaccharide production was fifteen times greater by galactose-grown cells than glucose-grown. Some deficiency in sugar metabolism was indicated.

Cells of the mutant were grown in minimal medium containing glucose and containing galactose at 34° for 15 hours. The specific activities of the enzymes hexokinase, UDPGal-4-epimerase and UDPG pyrophosphorylase were compared with those of A4 cells grown in minimal medium containing glucose. The results are shown in Table 22.

Although the levels of the enzymes hexokinase and UDPGal-4-epimerase did not differ much between A4 and the mutant grown on glucose and galactose, the level of UDPG pyrophosphorylase differed considerably. In galactose-grown cells the activity was about 1/10th that of A4 and in glucose-grown cells hardly any activity was found. An additional assay was performed in which all the components of UDPG pyrophosphorylase assay were present except the substrate UDPG. It was replaced by an equivalent amount of UDPGal. The assay measured a combination of UDPG pyrophosphorylase and UDPGal-4-epimerase activities. In A4 the specific activity was 15, and in the mutant no activity was found.

The mutant appeared to be defective in the enzyme UDPG pyrophosphorylase. The enzyme UDPGal pyrophosphorylase which catalyses the synthesis of UDPGal from gal-1-P and UTP, has not been reported in K. aerogenes or E.coli strains although it has been found in calf liver (Ting and Hansen, 1968). If such an enzyme were present in the mutant, the results may be explained, as during growth on galactose, UDPGal would be formed. By epimerization UDPG would also be synthesized. However if the cells were grown on glucose, little or no UDP glucose or UDP galactose would be formed and thus no extracellular polysaccharide. However the enzyme UDPGal pyrophosphorylase may not be present in K. aerogenes

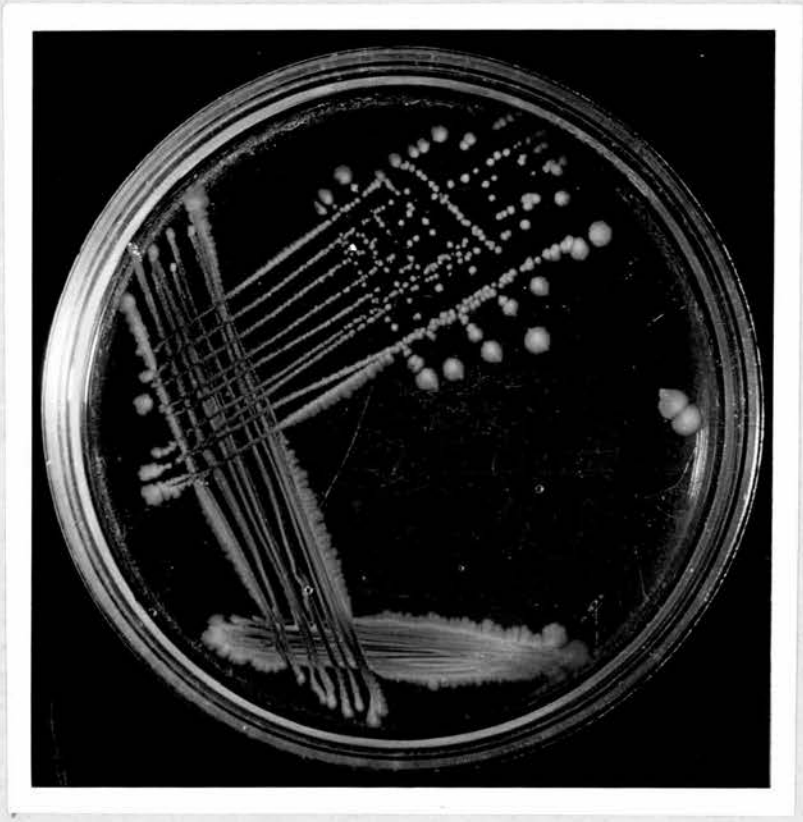


Plate 7. Mutant of K. aerogenes A1, non-capsulate and histidine-requiring, grown on yeast extract medium at 30° for 48 hours

strains. In this case a mutant lacking UDPG pyrophosphorylase would not be able to use galactose as a source of energy for growth. However the mutant of A4 grew in the presence of either galactose or glucose as sole source of energy producing acid and gas. In addition more extracellular polysaccharide was formed after growth on galactose than on glucose. The defect in the enzyme UDPG pyrophosphorylase may be leaky or revertants to wild type may be thrown off at high frequency. The intermediary metabolism between glucose and galactose in E.coli is shown in Fig. 29 (Fukasawa, Jokura and Kurahashi, 1963). If the pathways occur similarly in K. aerogenes, then during growth on glucose or galactose, if UDPG pyrophosphorylase is completely absent, no UDPG or UDPGal will be produced. The enzyme was present at a low level in the mutant culture. During growth on galactose sufficient UDPG and UDPGal may be formed to allow synthesis of extracellular polysaccharide, whereas during growth on glucose this may not have occurred. It was thus not known why polysaccharide production using glucose or galactose as carbon source differed to the extent found if it is assumed that the enzyme UDPGal pyrophosphorylase was absent and that the defect in UDPG pyrophosphorylase would be unchanged by growth of the mutant on glucose or galactose.

b) A further attempt was made to find sugar-deficient mutants by obtaining auxotrophs of K. aerogenes A1 which were histidine-requiring. In Salmonella strains one set of R mutants mapped at a locus, rfb, near "his" (Subbaiah and Stocker, 1964). These rfb mutants were unable to synthesize O-side chains often due to some defect in sugar metabolism. The region has been mapped in considerable detail using histidine-requiring mutants with deletions extending for varying lengths into it (Nikaido, Levinthal, Nikaido and Nakane, 1967).

TABLE 23.

Levels of enzymes in histidine-requiring mutants of A1

	non-mucoid mutant	mucoid unstable mutant	A1S1
glucose-6-P dehydrogenase	130	134	123
hexokinase	11.8	14	16.2
phosphoglucose isomerase	56	123	123
phosphomannose isomerase	11.2	31	28
UDPG pyrophosphorylase	22.4	30	28
GDPM pyrophosphorylase	2.3	4.7	5.6
UDPG dehydrogenase	0.06	0.11	0.11
GDPfucose synthetase	-	0.11	0.17

If mapping were similar in K. aerogenes strains, then histidine-requiring mutants, which are in addition non-mucoid, may be sugar-deficient.

Using penicillin selection techniques, histidine-requiring mutants of A1 were found at low frequency. Double mutants unable to synthesize the capsule occurred only very infrequently. A culture of one is shown in Plate 7. Another mutant was found which had two colony forms. The first was typically mucoid like A1 and the other non-mucoid with a rough appearance. The non-mucoid form was stable. When the mucoid form was re-grown in nutrient broth and plated out, a mixture of both colony types was produced. Indeed some colonies were a mixture of the two types being mucoid in the centre with spreading non-mucoid edges.

The enzyme levels of the mucoid unstable form and the non-mucoid form of the histidine-requiring mutant were compared to that of A1S1 after growth in yeast extract medium at 34° for 15 hours. The specific activities of the enzymes are shown in Table 23.

All the enzymes were present in the histidine-requiring mutant forms and their specific activities were very similar to those of A1S1. Thus in the case of the non-mucoid histidine-requiring mutant, the mutation did not involve a structural gene coding for the enzymes synthesizing the nucleotide sugars. In Salmonella strains, the rfb region also included the genes coding for transferases and some polymerases, so one of these enzymes may be affected in this mutant. The extracellular polysaccharide of the mucoid form was prepared and purified. It contained the same three component sugars, glucose, fucose and glucuronic acid, in the same proportions as the polysaccharide of A1. The change therefore was an "all-or-none" phenomenon, the cell either producing the A1 exopolysaccharide or not producing any. The mutant was however much more liable to throw off non-mucoid variants than A1. It was not known what change had taken place within the

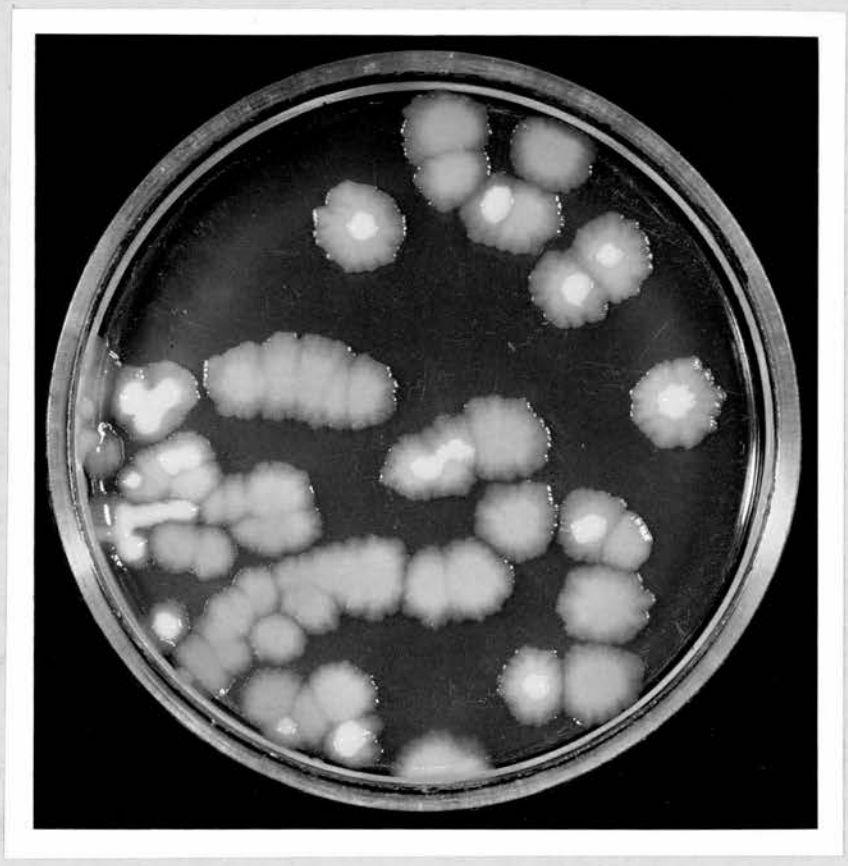


Plate 8. An unstable mutant of K. aerogenes A1 grown on yeast extract medium at 30° for 72 hours

genetical composition of the cell to cause this instability.

An additional unstable mutant of A1 was found which was not histidine-requiring. A1 was grown in nutrient broth containing 5×10^{-3} M pFA and dilutions spread over yeast extract medium. The appearance of one colony was quite different from the others. The centre of the colony contained cells synthesizing extracellular polysaccharide and was raised and shiny. The edges of the colony, however, were rough and spreading and contained non-mucoid cells. The characteristic appearance of this mutant is shown in Plate 8. If cells from the edges of the colonies were re-grown, only non-mucoid colonies were found. If cells from the centre were used, about half the colonies became non-mucoid and the other half were of the parent type with mucoid centres and non mucoid edges. Growth in the presence of pFA did not induce the non-mucoid colonies to become mucoid. As with the histidine-requiring unstable mutant of A1 it was not known what mutation in genetical composition or cellular change had taken place.

In E.coli an unstable mutant of "lac" was described (Schwartz, 1965). Colonies of this mutant were variegated producing sectors fermenting lactose and others not fermenting lactose. The cells not fermenting lactose were stable, while the lactose-fermenting cells were unstable producing variegated colonies as before. Acriflavine enhanced segregation of cells unable to ferment lactose from the unstable mutant, indicating that the unstable suppressor of the "lac" gene may be carried on an episome. The unstable mucoid mutant was grown with and without acriflavine. The % number of non-mucoid colonies was estimated after plating out the cultures. Acriflavine did not alter this %. An episomal system such as was present in the "lac" operon of E.coli thus did not seem to be involved in the production of exopolysaccharide in K. aerogenes A1.

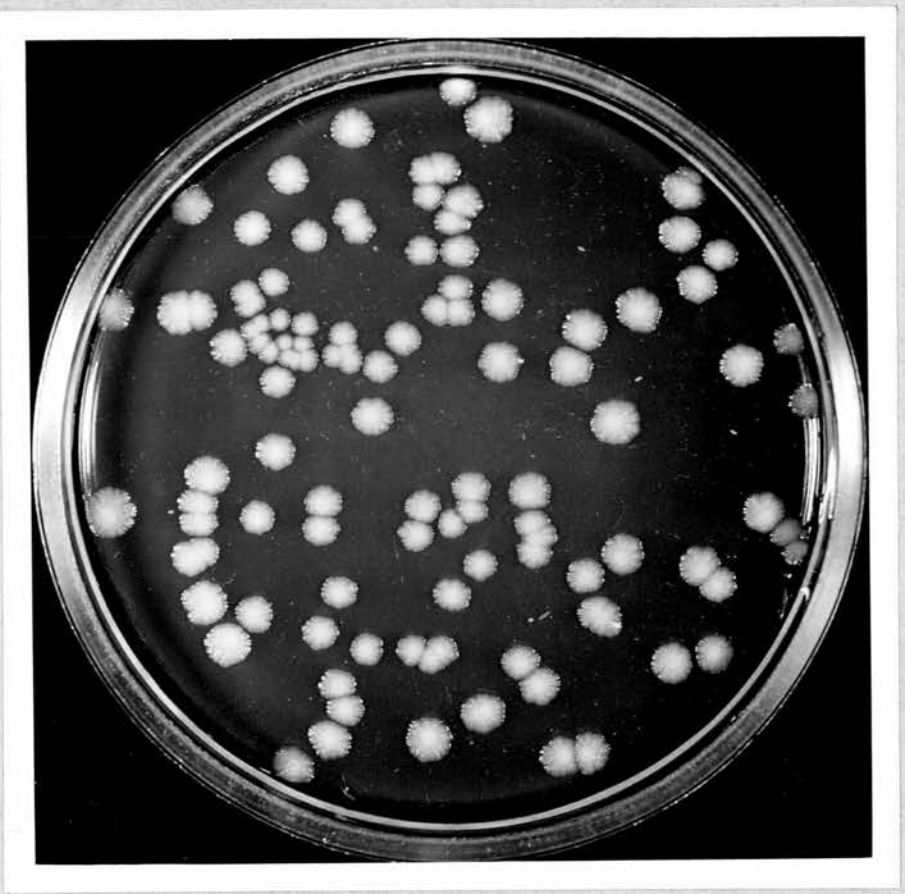


Plate 9. *K. aerogenes* A4CR grown on yeast extract medium at 20° for 72 hours

Plate 10. *K. aerogenes* A4CR (left) and A4 (right) grown in minimal medium at 20° for 48 hours

RESULTS

SECTION C

CR (Crenated) MUTANTS

A different type of exopolysaccharide synthesizing mutant of strain A4 called A4CR was found during examination of culture plates after mutagenesis. It was discovered following treatment of the culture with aminopurine on plates incubated at 30° or lower temperatures, and could be readily distinguished both from the parent strain and its non-capsulate mutant from its colony appearance at these temperatures. In size it was similar to A4(0) mutants but the colony surface was not smooth. Ridges radiated from a central apex and the outline of the colony was irregular. This characteristic appearance was most noticeable after incubation at 20°-30° for 24-48 hours. After longer incubation some mucoidness was noticed and the colonies adhered to the medium. On incubation at 37° the colony appearance was indistinguishable from that of A4. Plate 9 shows cultures of A4CR on yeast extract medium incubated at 20°. When grown on a balanced medium such as nutrient agar at 20°, the colonies of the mutant did not develop the crenated appearance so markedly and were sometimes difficult to distinguish from A4(0) mutants.

In liquid media at 37° as on solid media, the cultures of the mutant were indistinguishable from A4. There was uniform turbidity, and production of extracellular polysaccharide increased the viscosity of the medium. However if incubation was carried out at 20° the cultures of the mutant became autoagglutinable. A sediment of aggregated cells was formed if the culture was allowed to stand. At the same temperature a culture of A4 remained uniformly turbid and exopolysaccharide production occurred. Plate 10 shows the contrast between the mutant and A4 after growth in minimal medium at 20° for 48 hours. Autoagglutination was not prevented by altering the concentration of Ca^{++} or Mg^{++}

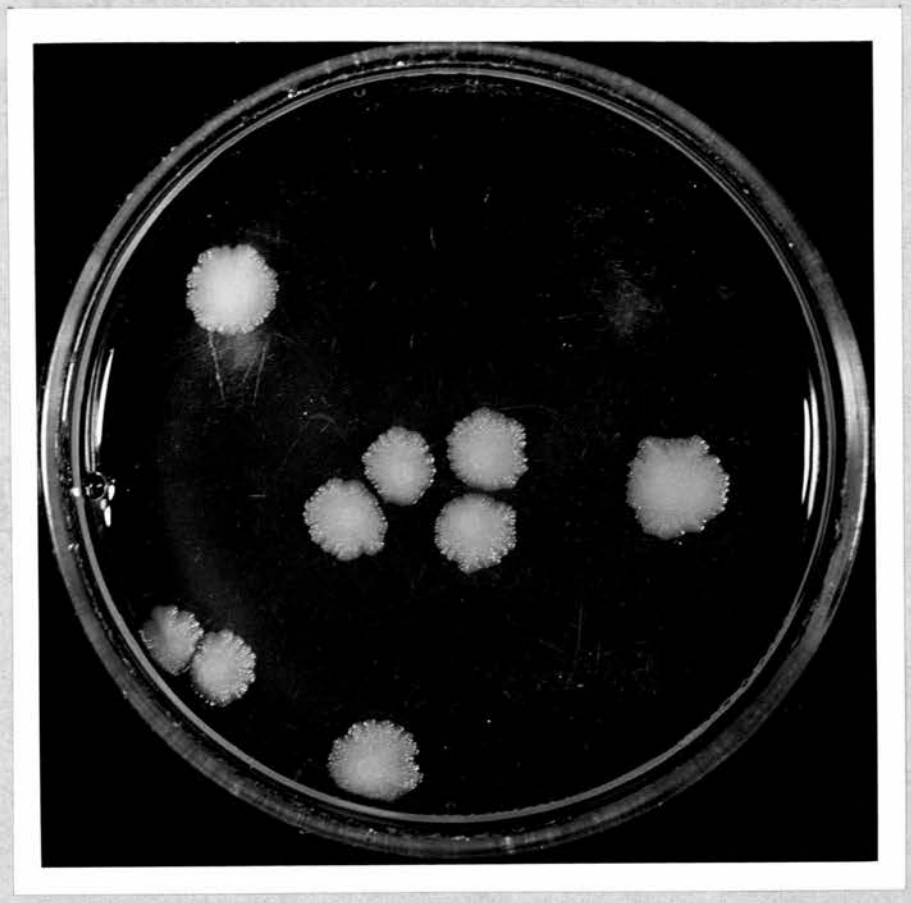


Plate 11. *K. aerogenes* A4CR(0) grown on yeast extract medium at 35° for 72 hours

Plate 12. *K. aerogenes* A4(0) grown on yeast extract medium at 35° for 48 hours

ions in the medium between 0 and 0.5mM or by adding EDTA up to 3mg/ml (w/v). Addition of Tween 80 in concentrations up to 1% (w/v) also failed to prevent autoagglutination. The source of carbon did not seem to matter, autoagglutination occurring using glucose, galactose, mannose or glycerol in a minimal medium.

No CR mutants of A1 or A1S1 were found, but CR mutants of strains A3S1 and W70 were discovered (Norval and Sutherland, 1969). These mutants were isolated only after treatment of the culture with aminopurine and not with other mutagens, except in one case where a CR mutant of W70 occurred spontaneously in an "old culture". The appearance of these CR mutants was identical to that described for the A4 mutant.

The A4CR strain was subjected to mutagenesis with aminopurine again and a further mutant form was isolated called A4CR(0). At 20° on solid media it had the characteristic appearance of the parent but was more friable. On prolonged incubation no mucoidness developed. At 37° in contrast to A4CR it retained the CR appearance, no mucoid material being produced. In liquid media the CR(0) mutant autoagglutinated at all incubation temperatures. The growth rate of the mutant was the same as A4(0) strains at 37° and 20° as measured by increase in dry weight. Plates 11 and 12 show the contrast between the A4CR(0) mutant and an A4(0) mutant.

An analogous CR(0) mutant was isolated from the CR mutant of strain A3S1 following treatment with aminopurine (Norval and Sutherland, 1969). Such a mutant of W70 was not found.

Mutation of the parent strains A4, A3S1 and W70 to the CR mutants using aminopurine as mutagen occurred at much lower frequency than mutation to non-capsulate mutants. Mutation of CR strains to CR(0) occurred very seldom. Using aminopurine as mutagen, A4 mutated to A4(0) at a rate of approximately 0.3%, and A4CR mutated to A4CR(0) at a rate of approximately 0.01%.

TABLE 24.

Reversion of A4CR to A4

Mutagen	Number of revertants	Total number of colonies	% rate of reversion
None (nutrient broth for 8 days)	0	1,500	0
aminopurine	2	600	0.33
acriflavine	1	300	0.33
uv	0	50	0
45°	1	2,000	0.05
EMS	4	1,000	0.4

REVERSION

As has already been noted, the A4(0) mutants were never found to revert to A4 despite the examination of over 10^4 colonies and the application of several mutagens. In a similar way A4CR(0) mutants never reverted to A4CR or A4. However A4CR cells were found to revert to A4 with fairly high frequencies depending to some extent on the mutagen used. Table 24 lists the rate of reversion using several mutagens.

No spontaneous revertants of A4CR occurred. However reversion of the CR mutant of A3S1 occurred spontaneously at a frequency of 0.01%.

PRODUCTION OF EXTRACELLULAR POLYSACCHARIDE.

When the CR mutants were first isolated, it seemed obvious that differences in extracellular polysaccharide synthesis from the parent strains occurred. The differences may be due to alteration in composition and structure of the polysaccharide. The composition of the polysaccharide of A4CR grown at 35° was determined. Like the parent strain it contained the three sugars, glucose, glucuronic acid and galactose in the ratio of 1: 1: 2. In addition, partial acid hydrolysates of A4 and A4CR polysaccharide were compared by paper chromatography andelectrophoresis and identical fragments were produced. Similarly the CR mutants of strains A3S1 and W70 have been shown to contain the same sugars in the same proportions as the parents (Norval and Sutherland, 1969). Phage-induced fucosidases active against the polysaccharide of A3S1 produced identical fragments when incubated with the polysaccharide from its CR mutant.

The difference in polysaccharide production was therefore not qualitative. Quantitative polysaccharide synthesis of A4CR at varying incubation temperatures on liquid and solid media was determined and compared to that of A4. Yeast

TABLE 25.

Extracellular polysaccharide synthesis (mgs polysaccharide/mg cellular protein)

Strain	Medium	Incubation Temperature 35° 24hrs	35° 48hrs	35° 72hrs	30° 48hrs	30° 72hrs	30° 96hrs	20° 72hrs	20° 96hrs
A4	Solid	2.8	3.5	3.0	1.6	2.0	2.8	3.7	3.7
	Liquid	2.1	2.3	2.4	2.1	1.9	2.0	2.2	2.2
A4CR	Solid	3.3	2.1	2.6	1.4	2.0	2.4	1.0	0.9
	Liquid	2.9	2.0	2.1	1.8	2.0	2.6	1.2	1.1
A4CR(0)	Solid or Liquid	0.1	0.1	0.1	0.1	0.05	0.05	0.05	0.05

TABLE 26.

Lipopolysaccharide of A4CR and A4CR(0)

	Incubation Temperature	LPS % dry cells	LPS Composition % glucose	% galactose	Glycogen % dry cells
A4CR	20	1.2	2.5	22.5	6.6
	35	2.1	3.7	20.7	6.0
A4CR(0)	20	2.57	1.6	17.9	5.4
	35	3.33	1.2	18.6	1.9

extract medium was used and the cultures were incubated at 20°, 30° and 37° for periods up to 96 hours. At intervals the cells were harvested and the capsular material removed by boiling. The cells were deposited by centrifugation and the polysaccharide in the supernatant estimated after dialysis. The results are shown in Table 25.

It was noticed that the greatest difference occurred when incubation had been carried out at 20°. The CR mutant synthesized only about 30% of that produced by the parent in liquid medium, and 50% in solid medium. The CR(0) mutant produced little or no polysaccharide so resembling non-mucoid mutants of the parent strain. Similar results have been obtained for A3S1, its CR and CR(0) mutants, and for W70 and its CR mutant (Norval and Sutherland, 1969).

The production of polysaccharide in CR mutants thus appeared to be temperature dependent. The function affected was not essential for growth over the range of incubation temperatures used.

Growth of A4CR in the presence of pFA did not induce production of polysaccharide and the colonies retained their characteristic crenated appearance when spread on plates containing minimal medium and pFA.

PRODUCTION OF LIPOPOLYSACCHARIDE AND GLYCOGEN

The lipopolysaccharide produced by the A4CR mutant was examined to find if its structure and composition was the same as the parent strain. The occurrence of autoagglutination and "roughness" in species such as E.coli and Salmonella has been associated with the production of incomplete lipopolysaccharide (Lüderitz, Staub and Westphal, 1966). The lipopolysaccharide of these groups contained approximately equal quantities of glucose and galactose in the basal structure.

TABLE 27.

Phage Sensitivity Patterns of Mutant and Wild Type Strains

	Incubation Temperature 37° Phage								Incubation Temperature 20° Phage							
Host Cells	31	33	36	37	38	40	41	42	31	33	36	37	38	40	41	42
A3(S1)	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-	-
A3CR	+	+	+	+	+	+	-	-	+	-	+	±	-	+	-	-
W70CR1	-	-	-	-	-	+	+	+	-	-	-	-	-	+	-	+
W70CR2	-	-	-	-	-	+	+	-	-	-	-	-	-	+	+	-
A3CR(0)	-	+	+	+	+	+	-	-	+	+	+	+	+	+	-	-
W70	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+
A4	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-
A4(0)	-	-	-	-	-	+	+	-	-	-	-	-	-	+	±	-
A4CR	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
A4CR(0)	-	-	-	-	-	+	+	-	-	-	-	-	-	+	±	-
Original Host	← A3S1 →								A1 A4 W70							

\+ = confluent lysis

\- = no lysis

± = very small number of plaques

The lipopolysaccharide of A4, on the other hand, has been shown to contain 17-23% galactose and only 2% glucose (Sutherland and Wilkinson, 1966) and this has been confirmed in strains A3S1 and NCTC 243 of K. aerogenes by structural studies (Koeltzow, Epley and Conrad, 1968).

The lipopolysaccharides of A4CR and its CR(0) mutant were prepared and purified after growth of the cultures at 20° and 35°. The yields and % composition of glucose and galactose were obtained and are shown in Table 26. The yield of lipopolysaccharide was lower following incubation at 20° than at 35° but the % glucose and galactose was the same at both temperatures. Similar results were obtained for the CR mutants of other strains also (Norval and Sutherland, 1969).

The surface properties of strains may be tested by their sensitivity to a range of phage preparation. The cultures were incubated in nutrient broth at 20° and at 37° and spread over the surface of nutrient agar plates. 0.02 ml drops of the phage preparation containing approximately 10^6 plaque forming units were applied and incubation continued at the two temperatures. There was no difference in phage sensitivity at the two temperatures. However if the strains were incubated through two subcultures in nutrient broth at 20° before being tested with the phage preparations, several differences in sensitivity were obtained from the pattern observed at 37°. The results for A4, A4(0), A4CR and A4CR(0) are shown in Table 27 together with the results from other CR strains.

Certain of the mutants seemed unable to synthesize the phage receptor at 20° or were unable to liberate the mature phage. The mutation causing a change in surface properties, also affects synthesis of the extracellular polysaccharide.

In some cases the mutants were sensitive to more phages than the parent which may reflect the protection of the cell surface by capsular material. It was not demonstrated in the A3S1 mutants where only slime was produced without a defined capsule.

A measure of the quantity of glycogen synthesized at the two incubation temperatures, 20° and 37°, by A4CR and A4CR(0) strains was obtained and is also shown in Table 26. The yield of glycogen did not show a distinct pattern. Similarily when other CR strains were examined there was considerable variation in the yield (Norval and Sutherland, 1969).

ENZYME LEVELS

Exopolysaccharide production occurs normally in the CR mutant at 37° and only at a slow rate at lower incubation temperatures. Also the yield of lipopolysaccharide is reduced at these temperatures. There may therefore be a conditional mutation affecting both exopolysaccharide and lipopolysaccharide synthesis. Such a mutation may be due to the alteration of an enzyme involved in synthesis of a nucleotide sugar required for both polysaccharide and lipopolysaccharide production. In strain A4 the polysaccharide consists of the sugars glucose, galactose and glucuronic acid, and the lipopolysaccharide of glucose, galactose, heptose, KDO and N-acetylglucosamine (Sutherland and Wilkinson, 1965). The sugars glucose and galactose are thus common to both the exopolysaccharide and the lipopolysaccharide.

The enzymes concerned in the synthesis of the nucleotide sugars UDPG, UDPGal and UDPGlUA in A4, A4(0), A4CR and A4CR(0) strains were assayed. The cultures were grown in minimal medium at 30° for 15 hours. The specific

TABLE 28

Enzyme levels of A4, A4(0), A4CR and A4CR(0) strains

	A4	A4CR	A4CR(0)	A4(0)
Hexokinase	49	51	28	33
glc-6-P dehydrogenase	11	20	78	21
phosphoglucose isomerase	8.5	22	56	78
phosphomannose isomerase	3.4	10	16	21
UDPGal-4-epimerase	0.79	1.0	1.6	2.0
UDPG dehydrogenase	0.11	0.11	0.17	0.24
UDPG pyrophosphorylase	5.6	7.8	13.4	39

TABLE 29.

Enzyme levels at various incubation temperatures

	A4		A4CR		A4(0)	
	22°	37°	22°	37°	22°	37°
Hexokinase	52	67	40	73	112	140
glc-6-P dehydrogenase	67	5.6	50	3.4	195	73
UDPG pyrophosphorylase	39	56	50	28	62	28
UDPG dehydrogenase	0.34	0.34	0.62	0.39	0.22	0.28
UDPGal-4-epimerase	3.9	0.45	1.6	0.11	2.2	0.90

activities are shown in Table 28.

The levels of most of the enzymes present in the A4CR cells were similar to the A4 cells. However the specific activities of glucose-6-P dehydrogenase, phosphoglucose isomerase and phosphomannose isomerase were raised 2-3 times in the CR mutant. Comparison of A4 with the A4(0) mutant showed these enzyme levels were raised also in the non-capsulate mutant and the CR mutant had activities midway between the A4 and A4(0) mutant. All the enzymes assayed were present in the CR(0) mutant and their specific activities were similar to the A4(0) mutant.

If there was a conditional mutation in one enzyme, a difference in specific activity after growth of the CR mutant at 37° and at 20° might be expected to occur. The enzyme levels of cells of A4, A4CR and A4(0) strains were therefore compared after growth at 22° and 37°. The strains were grown in minimal medium for 24 hours at the two incubation temperatures. The specific activities of the enzymes are shown in Table 29.

The levels of the enzymes hexokinase and UDPG dehydrogenase stayed approximately the same at both temperatures in all three strains. With regard to UDPG pyrophosphorylase, the level dropped in cells of A4CR grown at 37° compared with cells grown at 20°. However this drop also occurred in the A4(0) cells. The most striking difference in specific activity at the two temperatures was apparent in the levels of glc-6-P dehydrogenase and UDPGal-4-epimerase. In strains A4 and A4CR there was a drop of approximately 10 fold in the activity of the enzymes in the 37°-grown cells compared with 22°-grown cells. In A4(0) cells, the drop was about 2 fold. This lowering of activity in A4CR cells was paralleled by the parent strain, A4, and was therefore unlikely to account for the conditional

synthesis of polysaccharide in the mutant. The cultures had been incubated for 24 hours at the two temperatures which meant the 37°-grown cells were in late stationary phase while the 22°-grown cells may be only just into stationary phase. Whether the age of the culture may make a significant difference to the levels of enzymes in A4 was not determined. Such a difference was not found in strain A1S1. The presence of extracellular polysaccharide in the crude enzyme preparation may affect the specific activity of the enzymes, and, after growth of the culture at 37° for 24 hours, polysaccharide production may be greater than after growth at 22°. This is certainly true of the CR mutant. It may explain why the drop in enzymic activity of A4(O) cells was only 2-fold as this strain does not synthesize polysaccharide at either incubation temperature.

RESULTS

SECTION D

ATTEMPTS TO SYNTHESIZE EXTRACELLULAR POLYSACCHARIDE IN A CELL-FREE SYSTEM

Attempts to synthesize extracellular polysaccharide in cell-free systems have proved successful in a few instances only. In the genus Diplococcus a number of particulate preparations from disrupted cells synthesizing polysaccharides have been described (Mills and Smith, 1962). The exopolysaccharides hyaluronic acid (Markovitz, Cifonelli and Dorfman, 1959), colominic acid (Aminoff, Dodyk and Roseman, 1963) and cellulose (Glaser, 1958) have, in addition, been synthesized in cell-free systems. Nucleotide sugars labelled with C^{14} are utilised as glycosyl donors. The enzymic system is generally a crude particulate fraction obtained from cells broken by mechanical disintegration, ultrasonic treatment or EDTA-lysis. Attempts to solubilize the system have failed. Several methods have been employed to recognize the product formed as a result of the incubation of the nucleotide sugars with the enzyme system. The most specific of these utilize serological or enzymic techniques. Thus the polysaccharide synthesized in a cell-free system of Pneumococcus Type III is selectively precipitated with Type III antiserum and may be assayed immediately (Mills and Smith, 1962). Hyaluronic acid after its purification by chemical methods is assayed by use of the enzyme hyaluronidase (Markovitz, Cifonelli and Dorfman, 1959). Little, as yet, is known of any intermediates in the synthesis of these exopolysaccharides.

Although few systems have proved successful in the synthesis of exopolysaccharides, cell-free synthesis of the lipopolysaccharide of Salmonella and E.coli strains has been achieved in recent years. Often R-mutants, unable to

form a complete lipopolysaccharide, are used. Additions of sugars from the sugar nucleotide to the incomplete core structure may be obtained, as well as synthesis of O-haptens on a lipid carrier. Enzymic fractions are generally crude consisting of EDTA lysates (Robbins, Wright and Bellows, 1964) or cell envelope fractions (Rothfield and Horecker, 1964), both of which also contain acceptors for the synthetic system. Transferase enzymes are contained in the 105,000g supernatant fraction (Osborn, 1968).

K. aerogenes A4 was used in attempts to synthesize the extracellular polysaccharide in a cell-free system. The nucleotide sugars UDPG, UDPGal and UDPGlUA are believed to be precursors of the sugars in the exopolysaccharide. A specific method of detecting synthesis of the polysaccharide in the cell-free system was not found. Attempts to produce antiserum to the polysaccharide in mice failed. Also despite prolonged searches no bacteria or fungi were found which possessed depolymerases degrading the A4 polysaccharide. A wide range of samples were collected and selective media included that of Sickles and Shaw (1934), Torriani and Pappenheimer (1962), Barker, Pardoe, Stacey and Hopton (1963) and Waksman (1918). In all of these the source of carbon was an 0.01% solution of the A4 polysaccharide. The samples were sometimes inoculated first into a solution of the polysaccharide containing half-strength nutrient broth in case the depolymerase was inducible. Incubation was carried out at temperatures ranging from 15-55°. The presence of depolymerase would be indicated by a decrease in anthrone value, or appearance of reducing sugars as tested by Benedict's reagent. No culture of bacteria or fungi contained enzymes degrading the A4 polysaccharide.

Chemical methods were therefore used to detect the polysaccharide after

incubation in the cell-free system. These are therefore not so specific as enzymic or serological methods. At first water-phenol extraction of the system was carried out followed by electrophoresis. It was hoped that any polysaccharide or oligosaccharide synthesized would run only slowly or not move from the origin, thus permitting their separation from the nucleotide sugars. In later experiments the incubation mixture was chromatographed in Solvent IV. High molecular weight polymers remained at the origin while nucleotide sugars and other small molecules moved away. The origin material was then eluted and partially hydrolysed to obtain fragments of the polysaccharide. It was shown that hydrolysis for 15 minutes at 100° in $\underline{N}$ H_2SO_4 led to the production of three fragments, separable by electrophoresis. They were identified as the disaccharide glUA⟶gal, the trisaccharide glUA⟶gal⟶glc, and the tetrasaccharide glUA⟶gal⟶glc, with a further gal branching from glUA.

After electrophoresis the paper may be divided into strips and counted. Incorporation of radioactivity from a labelled nucleotide sugar into these hydrolysis products was therefore used to give a measure of polysaccharide synthesis.

Cells of A4 were grown in nutrient broth containing 1% glucose for 15 hours at 33° and the cell envelope fraction prepared. It was either used immediately or was stored at -20° and thawed slowly at 4° when required. Generally the fractions contained about 12 mgs protein/ml as measured by the method of Lowry $\underline{et\ al}$ (1951).

The typical reaction mixture contained 100µl 0.25 M Tris-HCl pH 7.8; 50µl 0.05 M $MgCl_2$; 5µl chloramphenical (2.5mg/ml); 100µl H_2O; 100 µl cell envelope fraction; 2µl 0.02 M UDPG; 4µl 0.01 M UDPGlUA; 8µl 0.01 M UDPGal; 10µl labelled nucleotide (10µl C^{14} - UDPGal contains 1.1 x 10^5 cpm; 10µl C^{14} -

TABLE 30.

Incorporation of radioactivity into lipid material (cpm)

Time (Mins.)	0	5	10	20	30	mμmoles incorporated/ 30 min/mg protein
C^{14}- UDPG	8	175	274	348	414	0.77
C^{14}- UDPGal	8	12	14	17	18	0.23
C^{14}- UDPGlUA	8	12	12	11	13	0.02

UDPGlUA contains 5.5×10^5 cpm; 10µl C^{14} - UDPG contains 3.7×10^5 cpm). Incubation was generally carried out at 25°. Incorporation of radioactivity into lipid material was determined by removing 20µl samples at intervals and extracting with a mixture of 2 mls $CHCl_3$ and 1 ml MeOH at 60° for 10 minutes. 1 ml saline was added and the two layers separated by centrifugation. The chloroform layer was evaporated to dryness and counted in the Triton-toluene scintillator. At the end of the incubation period the enzymes were inactivated by boiling for 1 minute and incorporation into the polysaccharide was determined as described above.

INCORPORATION INTO LIPID MATERIAL

Recently in the synthesis of three polysaccharides - the lipopolysaccharide of Salmonella and E.coli (Osborn et al, 1964), the mannose of M.lysodeikticus (Scher, Lennarz and Sweeley, 1968), and the murein of the cell wall (Higashi, Strominger and Sweeley, 1967) - lipid-linked intermediates have been shown to occur. Although such intermediates have not been reported during synthesis of exopolysaccharides except cellulose (Khan and Colvin, 1961) and recently that of a strain of K. aerogenes (Troy and Heath, 1968), this possibility has not been excluded. Lipid material soluble in $CHCl_3$/MeOH was therefore examined during incubation of the reaction mixture to ascertain if incorporation of radioactivity from labelled nucleotides occurred in the synthesis of the exopolysaccharide of A4.

Three tubes were set up containing the reaction mixture with a different nucleotide sugar labelled in each. At intervals over 30 minutes 20µl samples were removed, and incorporation of radioactivity from the nucleotide sugar into the lipid fraction was measured. The results are shown in Table 30.

TABLE 31.

Incorporation of radioactivity into lipid material (cpm)

Time (mins.)		0	30	60	90	120	180	240	mμmoles incorporated in 1st hour/mg protein
Temperature									
15°	C^{14}-UDPG	5	128	200	260	280	360	418	0.38
	C^{14}-UDPGal	0	7	12	21	15	19	21	0.15
	C^{14}-UDPGlUA	0	0	1	0	2	1	2	0.001
20°	C^{14}-UDPG	0	105	203	265	301	318	279	0.39
	C^{14}-UDPGal	0	7	13	15	-	20	21	0.16
	C^{14}-UDPG lUA	0	2	0	1	9	18	5	0
25°	C^{14}-UDPG	5	167	224	210	236	233	231	0.42
	C^{14}-UDPGal	1	14	19	34	20	18	17	0.24
	C^{14}-UDPGlUA	0	0	0	3	9	12	37	0

Labelled glucose from C^{14} - UDPG was incorporated into the lipid fraction quickly and to a considerable extent over the 30 minute incubation time. At 30 minutes, approximately 5% of the total radioactivity added was extracted in the $CHCl_3$/MeOH layer. Incorporation of labelled galactose occurred at a slower rate, and of labelled glucuronic acid hardly at all. In additional experiments it was found that no difference in the rate of incorporation of glucose occurred if UDPG was present without the other nucleotides, UDPGal and UDPGlUA, or with only one of them. Similarily incorporation of UDPGal and UDPGlUA occurred at the same rate without other nucleotides or with only one of them.

The incubation temperature was altered to ascertain if this affected incorporation rate. Incubation of the reaction mixture with one of the nucleotide sugars labelled was therefore carried out for a period of 4 hours at 25°, 20° and 15°. 20µl samples were taken at intervals and counts in the $CHCl_3$/MeOH layer are shown in Table 31.

When glucose was labelled, incorporation continued over the 4 hour period if incubation was carried out at 15°. At 20°, however, the count increased over the first 3 hours then fell off, while at 25° maximum incorporation was achieved after 1 hour. Thus if the normal period of the experiment was less than 60 minutes, it made little difference if 25°, 20° or 15° was used as the incubation temperature. Incorporation of galactose occurred more slowly at the three incubation temperatures. Hardly any incorporation of glucuronic acid at any temperature was found. Lowering of the incubation temperature thus did not seem to affect the rate of incorporation of these sugars into the lipid fraction.

Therefore glucose from UDPG and galactose from UDPGal were incorporated efficiently into lipid compounds and this occurred with or without the other

TABLE 32.

Effect of chloramphenicol on labelling of lipid material (cpm)

Time (mins.)	0	5	15	30	45	mμmoles incorporated hr/mg protein
chloramphenicol	0	13	28	52	67	0.16
no chloramphenicol	7	25	68	90	159	0.38

TABLE 33.

Effect of ADPG on labelling of lipid material (cpm)

Time (mins.)	0	15	30	45	60	mμmoles incorporated hr/mg protein
ADPG	3	33	70	126	144	0.27
No ADPG	0	88	130	185	229	0.44

nucleotides being present. Glucuronic acid from UDPGlUA was not incorporated to the same extent. Several possibilities were suggested from these results - lipid intermediates may not occur in the synthesis of the A4 exopolysaccharide; turnover of such intermediates may happen so rapidly that their presence cannot be detected in this system; glucose and galactose may be incorporated into lipid intermediates, the system not being suitable for incorporation of glucuronic acid; or the lipid-linked glucose or galactose compounds may be intermediates in the synthesis of a material other than the exopolysaccharide.

The lipid extractable material was examined to find if it contained any polysaccharide or oligosaccharide which became labelled in the reaction mixture. The reaction mixture contained the three nucleotides and C^{14}-UDPG, and incubation was carried out at 25°. At 0, 15 and 30 minutes the contents of one tube were extracted with $CHCl_3$/MeOH as before and the lipid fraction evaporated to dryness. The residue was partially hydrolysed in $\underline{N}$ H_2SO_4 for 15 minutes at 100° and electrophoresed to separate any charged material. The position of labelling on the paper was established by cutting it into sections and counting each section. No charged fractions were found and all the radioactivity remained at or near the origin. There was an increase in labelling of this origin material after 30 minutes compared to 15 minutes. None was found in the zero time sample. It was concluded that polysaccharide or oligosaccharide material attached to lipid either was not formed in this system or did not accumulate to an extent sufficient to be detected by the methods used.

The effect of chloramphenicol on the rate of incorporation of glucose from C^{14}-UDPG in the reaction mixture was found. Chloramphenicol is known to stop

TABLE 34.

Labelling of polymeric material (cpm)

	Time (mins.)	0	30	60	mμmoles incorporated hr/mg protein
I.	Pre-incubate with UDPG for 30 mins At time 0, add C^{14}-UDPGal After 30 mins, add UDPG1UA	17	227	356	8.2
II.	Pre-incubate with UDPG for 30 mins At time 0, add C^{14}-UDPG1UA After 30 mins, add UDPGal	160	433	765	1.4
III	Pre-incubate with UDPG + UDPGal for 30 mins At time 0, add C^{14}-UDPG1UA	116	403	652	1.2
IV.	Pre-incubate with UDPG + UDPG 1UA for 30 mins At time 0, add C^{14}-UDPGal	69	319	515	10.2
V.	At time 0, incubate with UDPGal, UDPG1UA and C^{14}-UDPG	180	4,120	5,834	19.6

TABLE 35.

Labelling of polymeric material (cpm)

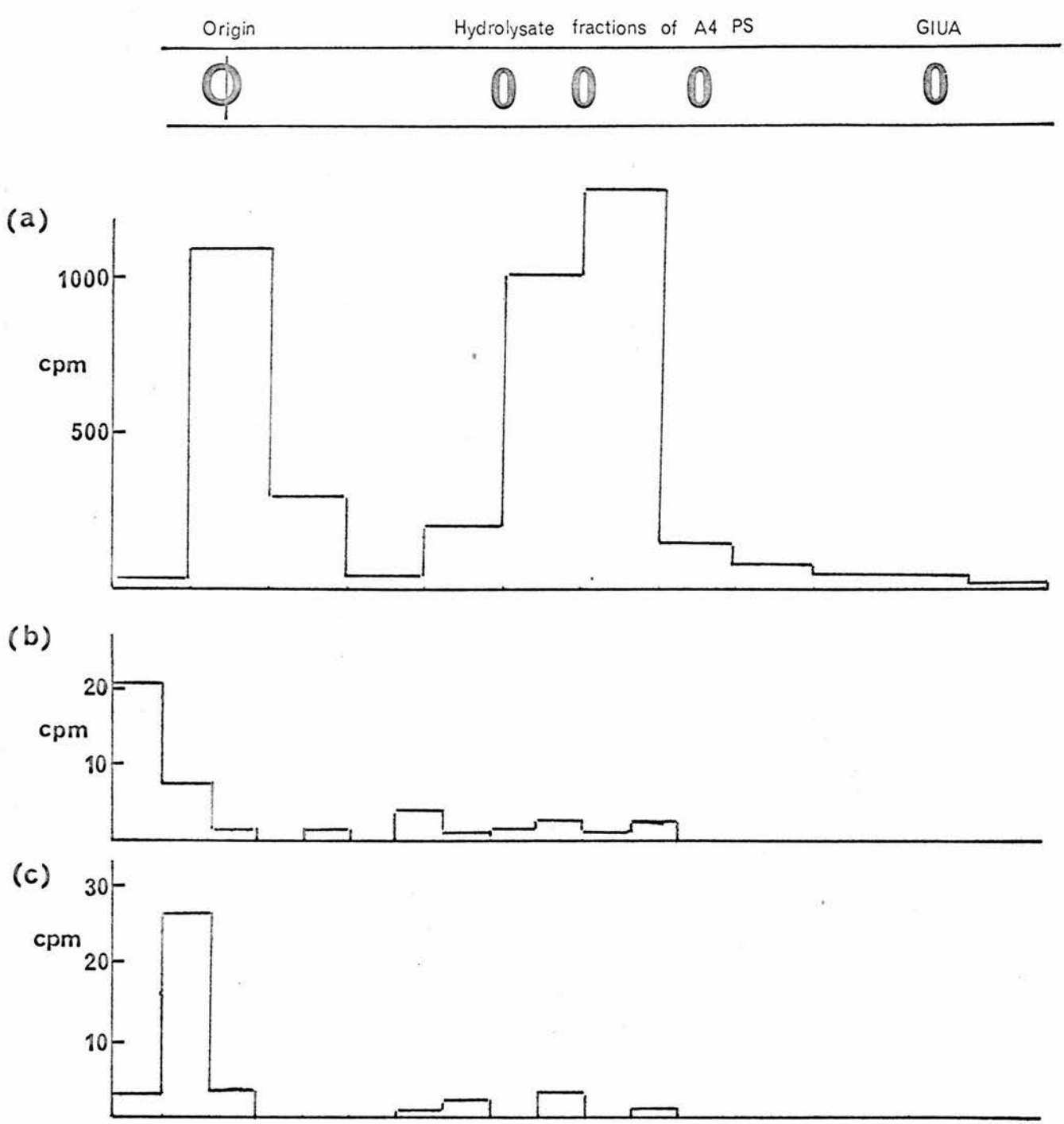
	Time (mins.)	0	2	5	10	20	30	mμmoles incorp./ 30 mins/ mg protein
VI.	At time 0, incubate with UDPG + C^{14}-UDPG1UA	200	470	527	-	1,406	1,737	3.5
VII.	At time 0, incubate with UDPG + C^{14}-UDPGal	8	69	209	286	514	558	12.7

protein synthesis by preventing incorporation of amino acids into protein. It was added to the reaction mixture to give a final concentration of 1 mg/ml. 20µl samples were removed at intervals throughout incubation and extracted with $CHCl_3$/MeOH. The labelling of the lipid extract is shown in Table 32. Thus incubation in the presence of chloramphenicol decreased the rate of incorporation of glucose into lipid extractable material by about 40%. It seems then that this rate is affected by the potential ability of the system to synthesize protein.

ADPG is used as a glucosyl donor for the synthesis of the intracellular polysaccharide, glycogen (Greenberg and Preiss, 1964), and is not known to be a glucosyl donor for the synthesis of other polysaccharides in the cell. The effect of ADPG on the rate of incorporation of glucose from UDPG into lipid extractable material was found. The reaction mixture was set up, one tube containing in addition to the 3 nucleotide sugars and C^{14}- UDPG, 10µl 0.01 M ADPG. Incubation was carried out at 22° and, at intervals, 20µl samples were removed and extracted with $CHCl_3$/MeOH. The results are shown in Table 33. The rate of incorporation of glucose from UDPG was decreased by 33% in the presence of ADPG.

ADPG, in addition to UDPG, was present in the nucleotide pool of A4. The cells were extracted with ethanol and the soluble pool fractionated on an Ecteola cellulose column. The fractions containing the bulk of the nucleotide sugars were pooled, freeze dried and electrophoresed. The presence of phosphate esters was detected by the method of Bandurski and Alexrod (1951). Spots corresponding to UDPG and ADPG were eluted and the glucose content determined after hydrolysis in 0.01N HCl for 10 minutes at 100°. ADPG and UDPG were both present and at almost equal concentrations. UDPGal and UDPGlUA were also found in the nucleotide pool.

FIG 30



Histograms showing position of labelling compared to hydrolysate fractions of A4 PS

(a) UDPG labelled

(b) UDPGIUA labelled

(c) UDPGal labelled

It seems difficult to explain the marked effect of ADPG on the incorporation of radioactivity from UDPG into lipid material. UDPG and ADPG are assumed to be specific for the synthesis of exopolysaccharide and glycogen respectively, and the enzymes used in the synthesis of one are not able to be used in the other. The pathways may not be entirely mutually exclusive however, and the availability of energy and any co-factors required may be altered if glycogen synthesis occurs in the same system as synthesis of extracellular polysaccharide. No lipid-linked intermediates have been found during de novo synthesis of glycogen from ADPG (Gahan and Conrad, 1968).

INCORPORATION INTO POLYSACCHARIDE MATERIAL

Uptake of labelled sugars from their nucleotide sugar derivatives into polymeric material was shown by incubation of the reaction mixture at 25°, taking 20µl samples at intervals and subjecting them to chromatography in Solvent IV. The origin material was then counted. The combinations of nucleotide sugars used and the counts obtained during incubation are shown in Table 34.

The greatest incorporation into the origin material occurred if glucose was labelled. However incorporation of galactose also occurred and glucuronic acid to a small extent. The results from tubes I and II showed that all three nucleotide sugars were not required to be present for labelling of the polymeric material to increase. This was confirmed in an additional experiment using further combinations of nucleotide sugars. These results are given in Table 35.

However these experiments served merely to show the incorporation of labelled sugars from their nucleotide derivatives into polymeric material, and not necessarily into exopolysaccharide material. Therefore the origin material, after chromatography in Solvent IV, was subjected to partial acid hydrolysis followed by electrophoresis. The paper was cut into sections and counted. Any labelling of oligosaccharide fractions was found by comparison with the fractions obtained

TABLE 36.

Labelling of component sugars in the exopolysaccharide.

Nucleotide sugar labelled	Origin	glucose		galactose		glucuronic acid	
	cpm	cpm	mμmoles incorp/ hr/mg protein	cpm	mμmoles incorp/ hr/mg protein	cpm	mμmoles incorp/ hr/mg protein
C^{14}-UDPG	0	153	0.028	255	0.046	31	0.006
C^{14}-UDPGal	0	46	0.056	23	0.028	0	0
C^{14}-UDPGlUA	0	5	0.0006	3	0.0004	11	0.0013

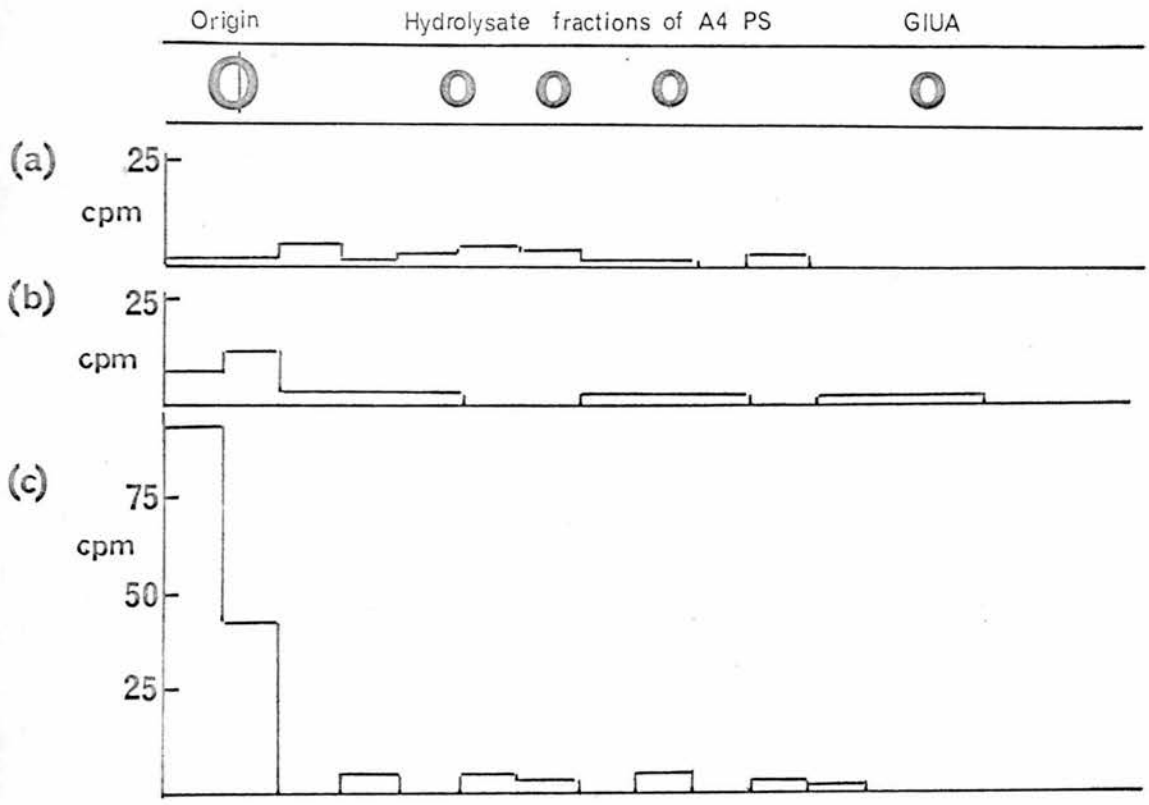
from partially hydrolysed A4 polysaccharide.

All three nucleotides were added to the reaction mixture, a different one being labelled in each case. Incubation was carried out for 1 hour at 25° before the polysaccharide material was extracted and partially hydrolysed. The histograms of the counts obtained are shown in Fig. 30 which also shows the relative positions of the hydrolysate fractions of A4 and glucuronic acid. When C^{14}-UDPG was used there was extensive labelling of the first two hydrolysate fractions and the origin material. No labelling of the fastest moving oligosaccharide was obtained. However if either C^{14}- UDPGal or C^{14}- UDPGlUA was used, little radioactivity occurred in the hydrolysate fractions. The same result was obtained if either UDPGal or UDPGlUA was the only nucleotide in the reaction mixture.

It was decided to test whether there was interconversion of the nucleotide sugars during the course of the incubation. If this occurred, the requirement for all three nucleotide sugars to be present before polysaccharide might be synthesized, would be overcome. The reaction mixture containing all three nucleotides, with a different one labelled in each case, was therefore incubated for 1 hour at 25°. After chromatography in Solvent IV, the origin material was completely hydrolysed in $\underline{N}$ H_2SO_4 at 100° for 16 hours. It was chromatographed in Solvent I and parts corresponding to the component sugars of the A4 polysaccharide were counted. The results are shown in Table 36.

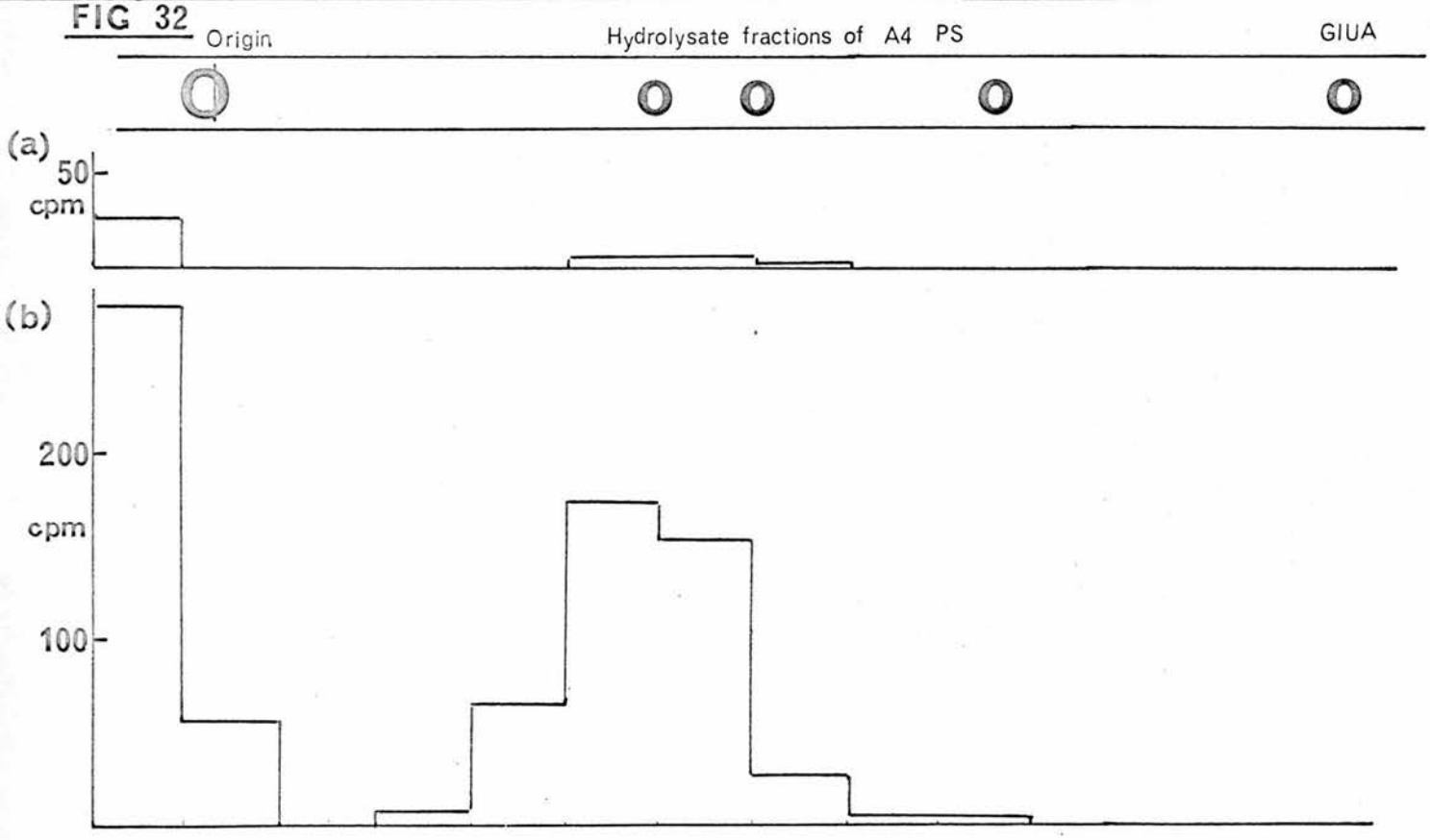
If C^{14}-UDPG was used, then the polysaccharide material contained labelled galactose and glucuronic acid, in addition to glucose, which showed that UDPG was converted to both UDPGal and UDPGlUA to a considerable extent during the course of the incubation. Similarily UDPGal was epimerized to UDPG although

FIG 31



Histogram showing position of labelling compared to hydrolysate fractions of A4 PS after use of an A4(O) cell-envelope: (a) UDPGIUA labelled
(b) UDPGal labelled
(c) UDPG labelled

FIG 32



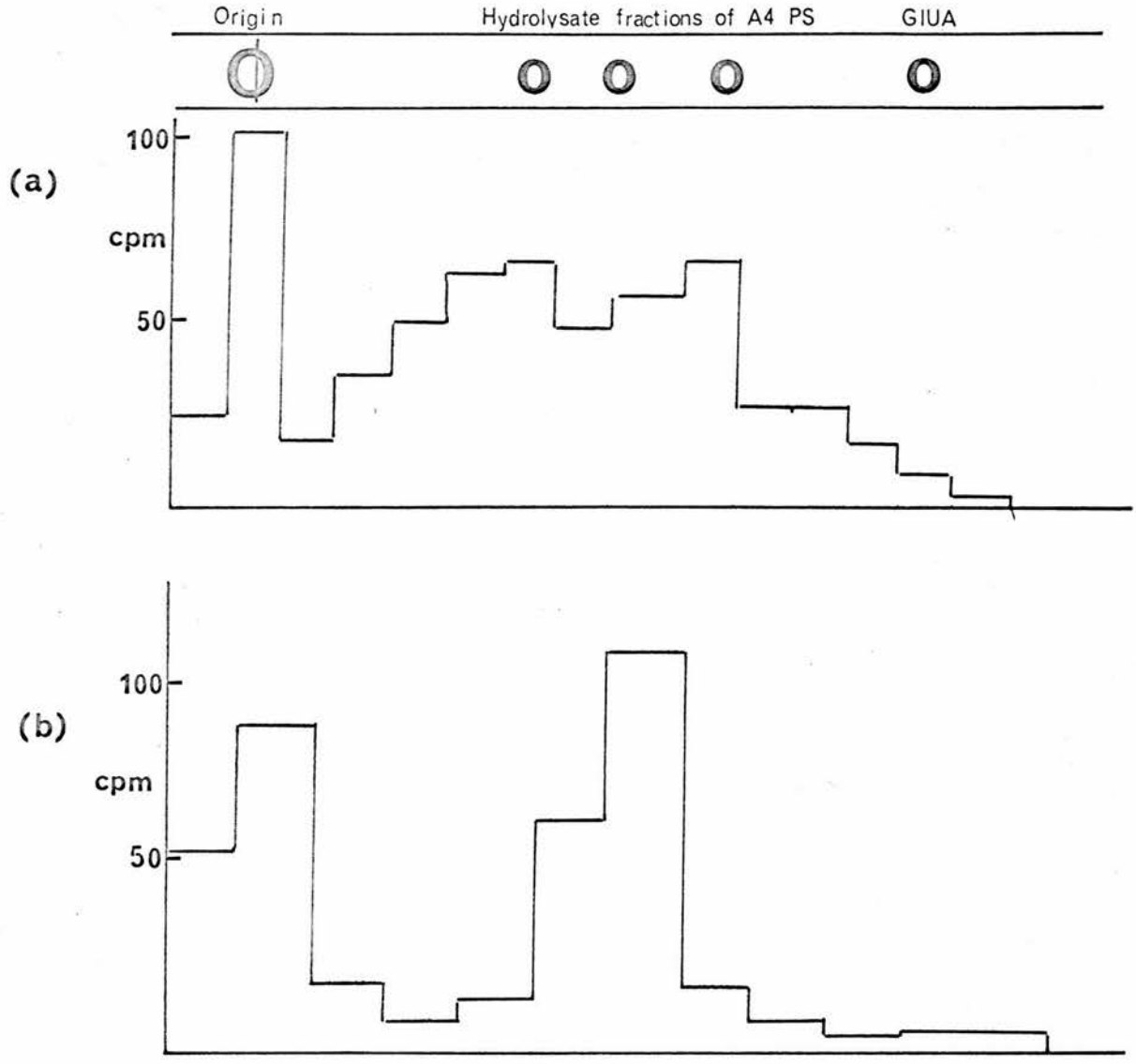
Histogram showing position of labelling compared to hydrolysate fractions of A4 PS using C^{14}-UDPG: (a) with chloramphenicol; (b) no chloramphenicol

UDPGlUA was not labelled. There was very slight labelling of all three sugars when C^{14}-UDPGlUA was used. If interconversion occurs during the incubation period then controls consisting of the reaction mixture without one or two nucleotide sugars, may still synthesize polysaccharide.

A non-capsulate mutant of A4 was used to provide the cell envelope fraction in the reaction mixture. The mutant was unable to synthesize extracellular polysaccharide under any circumstance. The same reaction mixture was set up as with A4, containing the three nucleotide sugars, a different one being labelled in each case. Incubation was carried out for 1 hour at 25°. Histograms of the counts obtained after partial acid hydrolysis are shown in Fig. 31. In the systems where UDPGlUA and UDPGal were labelled, little radioactivity occurred in the hydrolysate fractions or in the origin material. If C^{14}- UDPG was used, in contrast to A4, there was no incorporation of radioactivity into the hydrolysate fractions, although some incorporation into the origin material occurred. However total labelling was only about 10% of that obtained when A4 was used as the particulate enzyme fraction.

The experiment was repeated with three more non-mucoid mutants of A4 obtained by different mutagenic treatments. In every case, the results obtained were the same. This indicated that UDPG was used to synthesize extracellular polysaccharide material in A4 but not in A4(0) mutants. However it does not explain why in A4 little incorporation of radioactivity from C^{14}- UDPGal or C^{14}- UDPglucuronic acid occurred into the hydrolysate fractions. There was, of course, the possibility that, in fact, C^{14}-glucose was not being used in synthesis of new polysaccharide material but was exchanged for the glucose

FIG 33



Histogram showing position of labelling compared to hydrolysate fractions of A4 PS after pre-incubation with UDPG;

(a) UDPGIUA labelled

(b) UDPGal labelled

already in this structure. This seemed unlikely as glucose was shown to be on the main chain of the repeating unit of the polysaccharide.

The presence of chloramphenicol in the reaction mixture slowed down the rate of incorporation of glucose from C^{14}- UDPG into lipid material. Its effect on the incorporation of glucose into polysaccharide material was found. Incubation was continued for 1 hour at 25^{o} with and without chloramphenicol. Labelling of the hydrolysate fractions is shown in Fig. 32. The effect of chloramphenicol was striking. It stopped almost entirely the incorporation of glucose into the first two hydrolysate fractions. This showed the dependence of the system on the potential synthesis of protein material.

Incorporation of galactose or glucuronic acid from their uridine diphosphate derivatives into polysaccharide material, as indicated by labelling of the hydrolysate fractions, did not occur to any measurable extent. Pre-incubation with UDPG was tried. The reaction mixture was set up without nucleotides and incubated for 30 minutes with UDPG alone at 22^{o}. After this time the other two nucleotides, one of which was labelled, were added and incubation continued for a further hour. Labelling of the hydrolysate fractions is shown in Fig. 33. Labelling of these hydrolysate fractions occurred to an extent considerably greater than if the reaction mixture had been used without pre-incubation with UDPG. However total labelling is still below that obtained when C^{14}- UDPG was used. In the case of C^{14}- UDPGlUA, all three hydrolysate fractions were labelled in addition to the origin material. With C^{14}- UDPGal, the two slowest moving fractions and the origin material were labelled and the fastest moving fraction to a much lesser extent. It seemed then that if pre-incubation with UDPG was carried out before addition of labelled UDPGal or UDPGlUA then the reaction

TABLE 37.

Incorporation into hydrolysate fractions (di-, tri-, and tetrasaccharide)

	Total cpm	mμmoles incorp/mg protein
C^{14}-UDPG from Fig. 30(a)	2,740	0.5
C^{14}-UDPGal from Fig. 33(b)	211	0.25
C^{14}-UDPGlUA from Fig. 33(a)	374	0.045

mixture was able to synthesize a small quantity of extracellular polysaccharide material.

The area under the histograms in Figs. 30(a), 33(a) and 33(b) representing the tetra-, tri- and disaccharide fractions were integrated and the results are shown in Table 37. Glucose was incorporated to the largest extent. Incorporation of galactose occurred at about half this rate and of glucuronic acid about one-tenth. The result was paralleled by the incorporation of each sugar into lipid material.

DISCUSSION

All naturally occurring strains of K. aerogenes are mucoid and capsulate and they have been classified into at least 57 immunologically distinct K types (Edwards and Fife, 1952). The capsules were envisaged originally to consist of complex highly branched polysaccharides (Aspinall, Jamieson and Wilkinson, 1956). A template mechanism was considered necessary within the cell in order that the specificity of sequence of the monosaccharides in these structures might be retained (Wilkinson, 1958). However after this hypothesis had been put forward, several capsular polysaccharides were shown to consist of simple repeating units, often tetrasaccharides. The number of enzymes required for the synthesis of the polysaccharide is thus much reduced. Also the properties of these enzymes alone may account for the specific structure of each polysaccharide without the need for a template.

In the species K. aerogenes the structure of the exopolysaccharide of strain A3S1 and the conditions necessary for its production were studied first (Dudman and Wilkinson, 1956; Duguid and Wilkinson, 1953). During the present work strains of A1 and A4 were used. A1 is identical antigenically to A3, both belonging to Type 54, although the capsule of A1 is much larger. The exopolysaccharide of A1 consists of the same monosaccharide components, glucose, glucuronic acid and fucose, in the same proportions as that of A3S1, but it does not contain acetyl groups. Probably the two polysaccharides differ in chemical structure. The polysaccharide of A1 may be composed of a repeating tetrasaccharide unit containing glucose, glucuronic acid and fucose in the ratio 2: 1: 1. In this case and if a template mechanism is not involved, the enzymes required for the synthesis of the polymer from the nucleotide sugar precursors

are four specific transferases, one for each sugar component of the repeating unit. In addition a specific polymerase may be needed. A4 belongs to a different antigenic type than A1 and its exopolysaccharide consists of different monosaccharide components. However it may similarily consist of a repeating tetrasaccharide unit containing the sugars galactose, glucose and glucuronic acid in the ratio 2: 1: 1. Again four specific transferases plus a polymerase may be involved in its synthesis.

At the present time, a template mechanism would seem to be unnecessary although coding could occur on an m-RNA strand using the specificity afforded by the nucleotide part of the nucleotide sugar precursors of the polysaccharide. However this would have to be in conjunction with the sugar component also, as often more than one of the nucleotide sugar precursors of the exopolysaccharide contain the same nucleotide. For example in A4, all three precursors, UDPG, UDPGal and UDPGlUA, are uridine derivatives. In protein synthesis a template mechanism has been demonstrated. Proteins, however, do not contain repeating units and if a template were not used the number of specific transferases required would be very large and probably beyond what the cell could manage to synthesize. Occasionally during protein synthesis an alteration in the structure of DNA leads to an alteration in sequence of amino acids in the protein. It may have, as a result, slightly different properties. As far as can be ascertained this does not happen during exopolysaccharide synthesis although phage conversion where the phage genome alters the structure of the O-side chains of the lipopolysaccharide has been demonstrated. For example in E group Salmonella, phages ϵ^{15} and ϵ^{34} convert the structure of the repeating units of the O-side chains (Robbins, Keller, Wright and Bernstein, 1965; Uchida, Robbins and Luria, 1963). The alterations

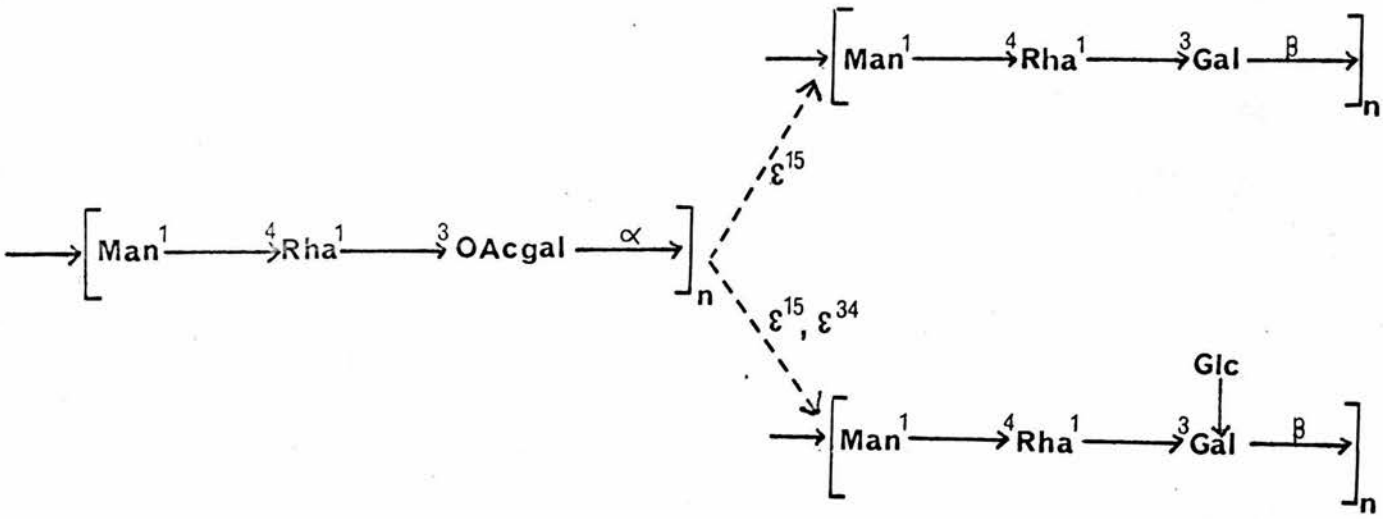


FIG 34

Alteration of O-side chains of Group E Salmonella by Phage

(Robbins and Uchida, 1963 ; Uchida, Robbins and Luria, 1963)

are demonstrated in Fig. 34. ε^{34} is thought to have a gene coding for a modifying enzyme, glucosyl transferase, so that glucose is added to the repeating unit. ε^{15} alters the unit in three ways, i) by producing a repressor inhibiting the synthesis of the α-polymerase of the units, ii) by producing a new polymerase so that the units are joined by a β-linkage and iii) by producing a repressor inhibiting the synthesis of the transacetylase enzyme. Modification of the exopolysaccharide, on the other hand, has never been shown. It is either produced or it is not produced. One sugar is never missing, or one replaced by another, and no differences in chemical structure have been demonstrated.

Specific transferases are involved in the synthesis of the core structure of the lipopolysaccharide of E.coli and Salmonella. The lipopolysaccharide is considered to be part of the cell wall and thus, like the exopolysaccharide, external to the cell membrane. Mutants have been isolated which lack one or other of the transferases, in which case the core structure is incomplete and the O-side chains cannot be joined on. The core, however, is not like the O-side chains or the exopolysaccharide because it consists of only four or five sugars and not a repeating unit. The O-side chains, on the other hand are similar in structure to the exopolysaccharide as they contain repeating units of about three or four monosaccharides. Their synthesis has been elucidated in great detail since the discovery that intermediates of the pathway were lipid-linked (Wright, Dankert and Robbins, 1965; Weiner et al, 1965). The repeating unit is built up on a lipid carrier by the sequential addition of monosaccharides. Polymerization then occurs followed by transfer of the O-side chain to the core structure. Such a lipid intermediate has not been reported until recently (Troy and Heath, 1968) to be involved in exopolysaccharide synthesis, but it seems

a hypothesis worth investigation.

In normal K. aerogenes strains extracellular polysaccharide is produced in the form of a defined capsule and, in addition, some is present as slime. Three classes of mutants affecting capsular production have been demonstrated.

1) Those mutants in which the ability to synthesize a defined capsule is lost and all the exopolysaccharide is present as slime.
2) Those mutants in which the ability to synthesize any exopolysaccharide is lost.
3) Those mutants in which the ability to synthesize exopolysaccharide is conditional, depending on the incubation temperature.

1) Slime-forming mutants

A mutant of A1 was found which synthesized extracellular polysaccharide entirely in the form of amorphous slime and not as a defined capsule. An analogous slime-forming mutant of the capsulated A3 strain has been described previously (Wilkinson, Duguid and Edmunds, 1954). From both the slime-forming and capsulate strains, mutants unable to synthesize extracellular polysaccharide are produced. The S1 strain does not seem to revert back to the capsulate parent strain although this change is difficult to screen, the appearance of colonies on solid media being similar. A possible method is to use a phage which selectively attacks non-mucoid or slime-producing variants only, and not the capsulated form. One such phage with these properties has been demonstrated in the colanic acid system.

The polysaccharide of A1S1 appears to be identical to that of A1 as regards component sugars and their proportions. In addition, levels of enzymes concerned in the synthesis of the nucleotide sugar predursors are similar. The exopoly-

saccharide of the capsulate A3 and A3S1 strains also appear to be identical both from the result of chemical analysis and from the action of phage depolymerases. Similarily colanic acid, the M antigen of Salmonella and E.coli strains, may be produced as capsules or slime and specific depolymerases act on both (Grant, 1968). It was thought that the properties of the polysaccharide may be affected by the % acetyl groups present. However the capsular and slime polysaccharide of A3 and of colanic acid both contain the same proportion of acetyl groups. The exopolysaccharides of A1 and A1S1 contain no acetyl groups and no other additional groups, as far as is known, which might affect the form of the polysaccharide.

As the change to slime production involves no easily detectable change in secondary polysaccharide structure, a change in tertiary structure may occur. The exopolysaccharide may be held in the capsule form on the surface of the cell by covalent bonds linked to the underlying cell wall. These bonds need be only very few per polysaccharide molecule. Alternatively it may be held on the surface by ionic bonds between acidic and basic substances at the cell surface, or mediated by divalent bonds (Rogers, 1965). Mutation to a slime-producing strain may result from a change in this organisation or a change in tertiary structure so that fewer groups are available for covalent or ionic bonds (Wilkinson, 1958). Alternatively there is the possibility that the polysaccharide itself may be unaltered, the mutation causing a change in structure or organisation of the cell wall so that the ability to form the polysaccharide into a capsule on the cell surface is lost.

2) Non-Mucoid mutants

Mutation from capsulate A1 and A4 or from slime-forming A1S1 strains to cells

not able to synthesize any exopolysaccharide occurs spontaneously at a low rate and at a higher frequency if a mutagen, such as acriflavine, uv light, high temperature of incubation, 2-aminopurine, is used. The rate of mutation induced by acriflavine or $CoCl_2$ is not higher than that using other mutagens. The control of the ability to produce exopolysaccharide does not therefore seem to be extrachromosomal. However reversion back to mucoidness is not obtained, either spontaneously or by use of several mutagens. This may indicate the irreversible loss of some chromosomal material.

A primer composed of partially hydrolysed exopolysaccharide was added to the plating out medium in case it was required as acceptor before synthesis of polysaccharide would begin but no revertants were obtained. There is the possibility that the primer consisted of molecules too large to penetrate the cell, so that suitable molecules would therefore not be present inside the cell to act as acceptor for the start of polysaccharide synthesis. Alternatively the primer may require to be lipid-linked. Indeed the likelihood of being able to add a primer to the culture in the correct form is remote. Using a cell-free system such an acceptor of 8-12 repeating units was required in the synthesis of Type III Pneumococcal polysaccharide (Mills and Smith, 1962). Similarily in cell-free synthesis of the extracellular polysaccharides, colominic acid, cellulose and hyaluronic acid, some polysaccharide was already present in the reaction mixtures which may act as acceptor. However against this, the O-side chains of the lipopolysaccharide of Salmonella and E.coli, consisting often of repeating tri- or tetrasaccharide units, may be synthesized without a primer or an acceptor (Zeleznick et al, 1965). Similarily one strain of K. aerogenes with a defect in the enzyme UDPGal-4-epimerase and thus unable to synthesize extracellular polysaccharide, forms the polymer in a cell-free system if the nucleotide sugars

UDPGal, UDPGlUA, and GDPMan are added (Troy and Heath, 1968). This is the only instance so far reported where a primer molecule is definitely absent from the reaction mixture during synthesis of an exopolysaccharide. Unless some known enzyme is defective so that synthesis of the precursors is impossible then one cannot be sure that all the molecules which could act as primers or acceptors in a cell-free system are absent. Thus in so-called de novo synthesis of glycogen in K. aerogenes, glycogen was present within the cells used to prepare the crude enzyme extract, albeit in very small quantities (Gahan and Conrad, 1968). The need for a primer before reversion to mucoidness is therefore not entirely ruled out until purified cell-free systems are developed for strains A1 and A4 in which synthesis of the exopolysaccharide occurs when the complete absence of compounds which could act as primers is known.

Following the growth of several non-mucoid strains of A1 in the presence of a phage specific for A1, mucoid revertants were obtained at low frequency. The phage may act as a partial selective agent for mucoid cells and indeed the capsule was shown to protect the cell to a certain extent from attack by phage. However the frequency with which mucoid revertants arose was rather high even if there was some selection of mucoid cells. Recombination, through transduction by the phage, may have occurred leading to the ability to synthesize a capsule. Recombination by transformation has been demonstrated in several Pneumococcal species (Mills and Smith, 1962). For example DNA from capsulated Type I cells transformed non-capsulated Type III cells, and capsules of Type I and a few of Type I-III were synthesized.

The mucoid revertants were immune to further attack by the same phage. In one revertant virulent phage particles were induced by uv light but not in the other revertants. Revertants were produced after incubation of the cells with

phage grown in a culture of A1(0) cells as well as A1 cells. If there was repair of the host chromosome by recombination, then it would not be expected that phage, prepared by growth with cells not themselves having the ability to produce a capsule, would act in transduction. The phage would not contain the part of the host genome required, unless different genes were necessary in the non-mucoid mutants used from that lacking in the non-mucoid mutant on which the phage was grown. This may explain why revertants were found in only about half the non-mucoid strains tried whether the phage had been grown on A1 or A1(0) cells. The polysaccharide produced by these revertant cells was the same, as far as could be ascertained, as the polysaccharide of A1 indicating there had been no change in genetical composition such as occurs in phage conversion of lipopolysaccharide structure. In addition no curing by acriflavine was demonstrated, so that the phage chromosomal material may be integrated into the chromosome of the host cells.

The levels of nine enzymes engaged in the synthesis of the nucleotide sugar precursors of A4, A1 and A1S1 exopolysaccharides were assayed in each of the non-mucoid mutants. Out of a total of around forty mutants obtained by treatment of the cultures with various mutagens, none was deficient in the enzymes tested. This seems unusual if it is assumed that the mutagens are liable to cause deletions or to alter the cells' DNA content at different loci depending on their particular properties. Hence it would be unlikely for all the mutagens to act at one locus, say affecting synthesis of a transferase. However during assembly of the monosaccharide components of the polymer from the nucleotide sugar precursors, there may be four specific transferases, plus a polymerase enzyme required. The mutation may be at any one of these loci and thus be unable

to be mapped. In synthesis of the core polysaccharide of lipopolysaccharides, mutations affecting transferases and polymerases were found to occur more frequently than those affecting production of enzymes involved in synthesis of nucleotide sugars (Nikaido, 1968).

Although in a few cases the levels of some of the enzymes assayed in the non-mucoid mutants were lowered, they were never completely lacking. There was no uniform lowering of the activity of any enzyme in the mutants as might be expected had the synthesis of the exopolysaccharide been under the control of a regulator gene. In the colanic acid system, cap R was shown to regulate the levels of UDPGal-4-epimerase, phosphomannose isomerase and GDPfucose synthetase, their specific activities being higher in the polysaccharide-producing strains than the non-mucoid. The levels of glc-6-P dehydrogenase and phosphoglucose isomerase were unchanged. It was concluded that normally production of exo-polysaccharide was repressed by the substance synthesized by cap R. If the repressor was altered, then polysaccharide was formed and the levels of these three enzymes de-repressed (Kang and Markovitz, 1967[a]). Later another regulator gene in addition to cap R was shown to be involved in polysaccharide synthesis. Mutation at this locus which allows synthesis of colanic acid does not lead to elevation of any of these enzymes, so that de-repression of their synthesis is not necessarily equated to synthesis of polysaccharide (Markovitz, Sydiskio and Lieberman, 1967). Thus the control of colanic acid synthesis appears to be at the genetic level.

It has been suggested from a comparison in mucoid and non-mucoid strains of the levels of nucleotide sugar precursors and the levels of some of the enzymes involved in their synthesis that several of the genes may be grouped on an operon

(Grant, 1968). Most of the enzymes in the GDPMan - GDPfucose pathway together with UDPG dehydrogenase may be in one operon, controlled by one or more regulator genes. Genes concerned in the synthesis of UDPG and UDPGal, on the other hand, may be outside the operon as these nucleotide sugars are required for functions in the cell other than the synthesis of colanic acid. In strain A4, the nucleotides involved in the synthesis of the exopolysaccharide are assumed to be UDPG, UDPGal and UDPGlUA. Of these UDPGlUA is the only one involved solely in exopolysaccharide synthesis. Similarily in strain A1, of the three nucleotide sugars involved, UDPGlUA and GDPfucose are concerned solely in exopolysaccharide synthesis. From the results of enzymes assayed in the non-mucoid mutants of A1 and A4, no repression of the synthesis of enzymes such as UDPG dehydrogenase or GDPfucose synthetase appears to have taken place. De-repression by pFA also does not occur. Thus the synthesis of exopolysaccharide in these strains of K. aerogenes is not shown to be regulated by a gene product like the colanic acid system. Also whether the enzymes involved are synthesized by genes grouped together in one operon is not known.

It has been shown that eight of the nine enzymes concerned with the synthesis of the O-specific sugars in the lipopolysaccharide of S.typhimurium map in one operon called the rfb region (Nikaido, 1968). It is positioned next to the histidine locus and histidine-requiring R-mutants have enzymes with altered specific activities. Although some were absent altogether, others had intermediate activities which indicated that more than one structural gene may be involved in their synthesis. Thus one locus, say for the enzyme UDPG pyrophosphorylase, may map in the rfb region, while another may map elsewhere on the chromosome and be unaffected by mutation at the rfb locus. This may also occur in the exopoly-

saccharide system in K. aerogenes. There may be more than one locus for a particular enzyme especially one involved in more than exopolysaccharide production such as UDPGal-4-epimerase or UDPG pyrophosphorylase. Therefore a mutation at one locus may not lead to total loss of activity of that enzyme, its function being taken over by another gene mapping elsewhere. This may explain the lowered specific activities of one enzyme in some mutants. It may also explain why inability to form exopolysaccharide was never found to be due to the complete absence of an enzyme synthesizing the precursors of the polysaccharide.

The levels of the nucleotide sugars present in the soluble pools of A1S1 cells and those of its non-mucoid mutants were estimated. Where the strain is unable to synthesize polysaccharide it might be expected that the nucleotide sugars UDPGlUA, GDPfucose and UDPG would accumulate, or at least the first two, UDPG being utilized for many purposes other than exopolysaccharide synthesis. However no large accumulation of the sugars was shown. Feed-back inhibition may occur in these strains preventing the build up of large quantities of unwanted nucleotide sugars. On mutation there may be a slight accumulation of the nucleotide sugars at first. This may be sufficient to result in inhibition of the activity of one of the enzymes involved in its synthesis, thereby preventing any further accumulation. For example GDPfucose may inhibit either GDPMan pyrophosphorylase or GDPfucose synthetase, or both. Such feed-back inhibition has been demonstrated, as in inhibition of dTDPG pyrophosphorylase by dTDPRha (Bernstein and Robbins, 1965), or inhibition of CDPG pyrophosphorylase by CDPparatose (Mayer and Ginsburg, 1965).

Some mutants of S. typhimurium unable to synthesize colanic acid were found

to have low levels of UDPGlUA and indetectable GDPfucose (Grant, 1968). This was taken to indicate that some control of the system lay at the nucleotide sugar level. If it were at the transferase level, then these intermediates would not be at a lower concentration than in mucoid cells. In conjunction with this, the levels of enzymes involved in the synthesis of GDPfucose and UDPGlUA were lower in the non-mucoid mutant. Feed-back inhibition was therefore ruled out as a control mechanism and a regulator controlling an operon considered. In exopolysaccharide synthesis in K. aerogenes, a different mechanism is involved as levels of the nucleotide sugar precursors and enzymes involved in their synthesis did not vary from mucoid to non-mucoid strains.

Two further mutants were found during the course of this work apart from the ones unable to synthesize any polysaccharide. One was an unstable mucoid form of strain A1. During growth in liquid culture a mixture of mucoid and non-mucoid cells were produced, while colonies on solid media often consisted of mucoid centres with non-mucoid cells at the edges. It is not known what caused this instability. It seems to be a form variation perhaps indicating some genetic instability. A system involving a similarily unstable mutant of "lac" has been described (Schwartz, 1965). It was shown that a suppressor of the lac operon was genetically unstable. It was carried on an episome and when in the autonomous state was unstable, its replication or segregation at cell division being inexact. It was stabilized by integration into the chromosome at different sites. However such an episomal nature of a suppressor for mucoidness was not indicated as there was no enhancement of segregation of non-mucoid cells from the unstable mucoid variant during growth with acriflavine. The genetic configuration specifying this mutation appears to be inherently unstable and may represent a change in the properties of a particular transferase or polymerase, making them more liable to

be defective, or in a regulator gene if such a control system is present.

The second mutant type was a variant of strain A4. It produced only a small amount of exopolysaccharide when grown on minimal medium containing glucose and the normal amount, like A4, on minimal medium containing galactose. At first sight this could be explained by a defect in the enzyme UDPGal-4-epimerase. However the mutant grew on EMB-galactose medium forming dark galactose-fermenting colonies and also grew in galactose peptone water producing acid and gas. In addition the result of enzyme assays showed that UDPG-4-epimerase was present at the same level as in A4. However the enzyme UDPG pyrophosphorylase had only low specific activity, about 10% of the parent. A total block at this step would mean that no UDPG, and hence no UDPGal, could be formed either during growth on glucose or galactose. The enzyme UDPGal pyrophosphorylase has not been reported in bacteria. UDPG pyrophosphorylase is not inducible and, although the block in its activity was not absolute, it does not explain the large difference in polysaccharide production which occurs after growth on glucose or galactose as the carbon source.

Due to the lack of suitable selective methods, no mutants have been isolated synthesizing an incomplete exopolysaccharide. Indeed mutants such as these may never occur as the exopolysaccharide has been shown to consist of repeating units and a mutation affecting its synthesis may be an "all or none" phenomenon, causing complete loss of polysaccharide synthesizing ability. Mutants affecting lipopolysaccharide synthesis in which an incomplete core is formed have been recognised by the rough nature of the colony. Also others producing an incomplete O-side chain consisting of one repeating unit only have been recognised. In these one unit is added to the completed core structure but the enzyme polymerase necessary for the addition of the polymerized repeating unit is absent. A difference occurs in the exopolysaccharide structure in that no core is known so that the repeating unit polymer has no comparable acceptor. Thus whether an equivalent mutation could occur in exopolysaccharide synthesis and whether it

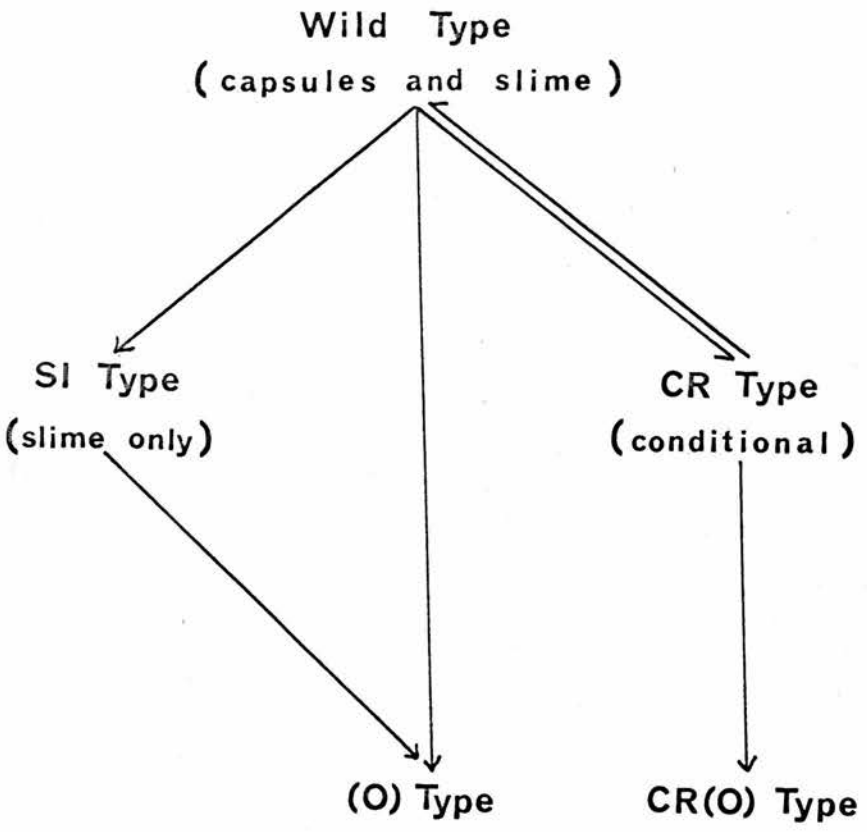


FIG 35

Scheme for Mutations affecting Exopolysaccharide Synthesis

would alter the appearance of the colony sufficiently to let it be distinguished from non-mucoid strains is not known.

3) CR mutants

The third type of mutant affecting exopolysaccharide synthesis is one in which production is temperature dependent. A number of temperature dependent mutants have been described and they can be divided into two classes. In the first, the function affected is essential for growth so that incubation at the normal optimal growth temperature is lethal. One such mutant of E.coli, unable to grow at 37°, had a temperature-sensitive amino-acyl RNA synthetase (Eidlic and Neidhart, 1965). In the second, the function affected is not essential for growth but is demonstrated only when the cells are cultured below the optimal growth temperature. Temperature sensitivity has been correlated in the cases studied with an altered enzyme protein or repressor (Böck, 1968; Kang and Markovitz,1967[b]; Jockusch, 1966). One surface antigen of E.coli, the f^+ antigen has been reported where production is like that of the exopolysaccharide in CR mutants. The antigen is lost at lowered incubation temperatures but is formed again on return of the cells to 37°. It is probably polysaccharide in nature (Knolle and Ørskov, 1967)

The CR mutants arise only rarely and much less frequently than non-mucoid mutants. Furthermore, they only arise spontaneously or under the influence of the mutagen aminopurine. They are less stable than the non-mucoid mutants and can revert back at low frequency to the parent strain either spontaneously or at a higher rate under the action of a mutagen. Also very rarely a double mutant occurs which has lost the ability to synthesize polysaccharide but retains the CR appearance. The mutations involving exopolysaccharide are illustrated in Fig. 35.

Assuming that no double mutation has taken place, the conditional mutation producing the CR strains appears to have two effects. The first is to reduce synthesis of exopolysaccharide at lowered incubation temperatures. This returns to the level of the wild type if the incubation temperature is raised. The second is to affect one of the surface components - probably the lipopolysaccharide. The structure of the lipopolysaccharide is not altered but its quantitative production is. The lowered lipopolysaccharide content leads to autoagglutinability of the cultures and their "rough" appearance.

There may be alteration of one of the enzymes involved in formation of the nucleotide sugar precursors of the lipopolysaccharide and exopolysaccharide, or an altered regulator. The lipopolysaccharides of the K. aerogenes strains used contain galactose, heptose, KDO and N-acetylglucosamine (Sutherland and Wilkinson, 1965). The exopolysaccharide of A4 contains glucose, galactose and glucuronic acid, that of A3S1 glucose, glucuronic acid and fucose, and that of W70 galactose, mannose and glucuronic acid. No sugars are therefore shared by the lipopolysaccharide and exopolysaccharides of all three strains. However as UDPG is involved in the formation of UDPGlUA and UDPGal, an alteration in its production could affect both the lipopolysaccharide and polysaccharide. Thus in the pathway shown below, alteration of any of the three enzymes would effect UDPG synthesis :

glucose ⟶ glucose -6-Ⓟ ⟶ glucose-1-Ⓟ ⟶ UDPG

hexokinase phosphoglucomutase UDPG pyrophosphorylase

The variations in hexokinase and UDPG pyrophosphorylase levels of A4 and its CR mutants at 37° and a low incubation temperature were compared. No difference was apparent. Phosphoglucomutase, however, was not assayed. The metabolic balance of the cell may be altered by the mutation. For example, ADPG is the

glucosyl donor for glycogen synthesis (Gahan and Conrad, 1968). If a reduction in glycogen synthesis occurs at low temperature together with a reduction in lipopolysaccharide and exopolysaccharide production, then this may indicate hexokinase or phosphoglucomutase are affected. However the results for glycogen yields were not consistent enough to determine whether a significant reduction had, in fact, occurred at lowered incubation temperatures.

The alternative to an alteration in the allosteric properties of an enzyme in CR mutants is an alteration in some regulator gene. The regulator may have the function of a repressor on exopolysaccharide synthesis being active at low temperatures only, or it may act as an inducer of polysaccharide synthesis at high incubation temperatures. However it would require to have the same action on the synthesis of lipopolysaccharide at the different incubation temperatures, and it would seem unlikely that one regulator could affect both lipopolysaccharide and exopolysaccharide synthesis in the same way. Again it may act on one of the enzymes common to the synthetic pathways such as hexokinase, UDPG pyrophosphorylase or phosphoglucomutase.

The third possibility is that a double mutation has taken place leading to separate effects on lipopolysaccharide and polysaccharide synthesis. There may be alteration in two regulators or in the allosteric properties of an enzyme of each pathway. However reversion to the parent form takes place spontaneously or under the action of a mutagen quite frequently. One would not expect repair of a double mutation to occur so readily. In addition, if this were the case, revertants with repair of only one property should occur, such as a mutant with temperature-conditional synthesis of polysaccharide but with normal lipopolysaccharide production. At low temperatures the culture would synthesize only

small amounts of exopolysaccharide but would not be autoagglutinable or have a "rough" appearance. Such mutants were not detected. Any loss of the CR appearance was accompanied by a return of the ability to synthesize exopolysaccharide. Also further mutants of the CR strains arose which retained the CR appearance at all incubation temperatures and did not synthesize exopolysaccharide. This may be taken as a further indication that the two effects are not separable.

ENZYME LEVELS

The levels of enzymes in cells of strain A1S1 grown under various conditions were compared with the ability of the culture to synthesize extracellular slime. On a medium such as nutrient agar where the carbon and nitrogen content are relatively balanced, exopolysaccharide production is minimal. On the other hand in yeast extract or minimal media, polysaccharide production is much greater due to the large carbon excess. However the quantity of exopolysaccharide synthesized was not reflected by the specific activities of the enzymes concerned in the production of the sugar nucleotide precursors of the polysaccharide. There was no variation in the levels of these enzymes prepared from cells grown in different media. Furthermore the activity of the enzymes did not change after growth of the cells in media which altered their subsequent ability to synthesize exopolysaccharide in washed cell suspensions. For example, the culture was grown in a medium containing salts and casamino acids with no additional carbon source. The ratio of nitrogen to carbon was consequently high and the synthesis of exopolysaccharide low. In washed cell suspensions, these cells showed only slight ability to produce exopolysaccharide when compared to cells grown in a medium where the ratio of nitrogen to carbon was low. However the specific activities

of the enzymes assayed were unaltered.

It seems then that the enzymic machinery of the cell is available to allow synthesis of the nucleotide precursors of the exopolysaccharide at all times, and is not altered by the conditions under which the cells have been cultured. Some of these enzymes such as GDPMan pyrophosphorylase, GDPfucose synthetase and UDPG dehydrogenase may be concerned solely in the formation of the nucleotide sugars for exopolysaccharide synthesis. Where the cell is not producing polysaccharide due to environmental conditions the formation of these enzymes would appear to waste both energy and metabolic intermediates within the cell. If they were only formed by induction under circumstances in which some synthesis of the polysaccharide could occur, this would conserve energy and materials. In a similar manner, this wastage occurs in non-mucoid mutants, this time under all conditions, as exopolysaccharide is never synthesized.

Discussion of these points together with the three types of mutants leads to a consideration of the control of polysaccharide production in K. aerogenes. It has already been indicated that the specificity of the polysaccharide is more likely to be decided solely by the enzymes involved in its synthesis than by a template mechanism. The control of synthesis may be either at the metabolic level or the genetic level.

If it were at the genetic level, there may be a system such as has been described for colanic acid, where the majority of genes coding for enzymes involved in synthesis of the polysaccharide are grouped together in an operon controlled by a regulator either episomally or integrated into the chromosome (Markovitz, 1964), and lipopolysaccharide, where the majority of genes for O-antigen side chain synthesis are grouped together at one locus. In exopolysaccharide synthesis

an episomal system was not demonstrated although the inability of non-mucoid mutants to revert may be taken to indicate the irreversible loss of some chromosomal material. However no properties of an episome were shown such as curing by acriflavine or $CoCl_2$, or transmission of mucoidness to a suitable recipient cell. The control was, in addition, unlike that in the colanic acid system as de-repression of non-mucoid mutants by pFA did not occur. The gene product normally formed which represses colanic acid synthesis was sufficiently altered by growth in the presence of pFA to prevent its usual effect on the control of the operon. Finally control of exopolysaccharide synthesis is probably not genetic as there was no general lowering of the levels of enzymes involved solely in exopolysaccharide production compared to the parent strains, such as GDPMan pyrophosphorylase, GDPfucose synthetase and UDPG dehydrogenase in A1(0) strains, and UDPG dehydrogenase in A4(0) strains. Furthermore alteration of the activity of these enzymes did not occur in the parent strains, even when the quantity of exopolysaccharide synthesized by the cell varied considerably.

Control seems more likely to be at the metabolic level. In this case the concentration of the precursors and the activity of the enzymes involved in their production and degradation is important. The synthesis and breakdown of the intracellular polysaccharide, glycogen, is influenced by the presence of certain intermediates. For example, ADPG pyrophosphorylase is activated by compounds such as fructose-1, 6-di Ⓟ, glucose-1, 6-di Ⓟ, glyceraldehyde-3-Ⓟ and is inhibited by phosphate, AMP and ADP (Preiss, Shen, Greenberg and Gentner, 1966). This means that under circumstances where growth is limited, particularly by lack of nitrogen, DNA, RNA and proteins are required to a lesser extent than normal. ATP and some intermediates may then be produced in excess leading to stimulation of ADPG pyrophosphorylase and glycogen synthesis. A similar system may operate

in the regulation of exopolysaccharide synthesis, whereby the accumulation of a particular intermediate may stimulate the activity of some enzyme(s) involved in the pathway. In addition the level of the nucleotide sugars may affect the synthesis of exopolysaccharide. Where the nucleotide sugar is unique to the particular pathway, control may be exercised at this point. Feed-back inhibition preventing accumulation of intermediates in non-mucoid mutants may occur. The presence of such a mechanism was demonstrated in three non-mucoid mutants of AlS1 which showed no large accumulation of the nucleotide sugar precursors of the polysaccharide, despite the fact that they were unable to synthesize the exopolysaccharide. The absolute level of enzymes concerned in the synthesis of these nucleotide sugars did not vary according to the medium the cells had been grown on. This indicated that there was no induction of exopolysaccharide production and that only the activity of these enzymes or the availability of their substrates was affected.

To verify this conclusion it would be necessary to purify the enzymes involved in the synthesis of the nucleotide sugars and to find the effect of various intermediates and metabolites on their activity. For example the enzymes UDPG pyrophosphorylase and TDPG pyrophosphorylase of E.coli have been purified (Bernstein and Robbins, 1965). Both were constitutive, being present even if the cell did not synthesize exopolysaccharide. UDPG pyrophosphorylase was inhibited competitively by TDPG and TDPRha and was also inhibited by high levels of pyrophosphate. TDPG pyrophosphorylase was similarily inhibited by UDPG and TDPRha. It was concluded that negative feed-back at the levels of these enzymes may occur. Alternatively their activity may be controlled by the level of such precursors as TTP or glucose-1-Ⓟ. There may be similar control in

K. aerogenes strains, for example negative feed-back control by GDPfucose of the activity of GDPMan pyrophosphorylase in A1, or inhibition by UDPGlUA of UDPG dehydrogenase in A4.

There is also the possibility that some sort of negative control may be exercised by the presence of degradative enzymes breaking down the nucleotide sugar precursors or the co-factors involved in their synthesis. Thus CDPglycerol pyrophosphatase has been found in extracts of Lactobacillus which may be important in control of teichoic acid synthesis (Glaser, 1965). However other enzymes degrading sugar nucleotides have been detected and as they were found on the outside of the cell, their function may be to degrade the sugar nucleotides in the environment and make them available to the cell (Glaser, Melo and Paul, 1967).

Finally the activity of the transferase and polymerase enzymes may alter the rate of exopolysaccharide synthesis. This has been demonstrated in glycogen synthesis in animals and yeasts. Glycogen synthetase is stimulated by intermediates such as glucose-6-P (Rothman and Cabib, 1966). The glucosyl donor is UDPG and since it is also involved in many other reactions in the cell, control at this, the final stage of glycogen synthesis, would obviously be more definite than if control were at the level of UDPG formation. However glycogen is a homopolymer so that control may be assumed to be much simpler than in the case of the exopolysaccharides, which are heteropolymers and presumably require the sequential action of several transferases and a polymerase. In addition an intermediate stage such as a lipid-linked intermediate may occur. Thus control may be at an earlier level in the synthesis of the exopolysaccharides.

Exopolysaccharides are produced in largest quantities when growth is limited by lack of N,S or P but are still produced to some extent in all media. Once the

polysaccharide is formed it cannot be broken down again by the cell except in the case of hyaluronic acid of Streptococci. It thus differs in function from the intracellular polysaccharide, glycogen, which is formed under similar conditions but when normal growth starts again or nutrients are not available, it may be degraded, releasing glucose molecules and energy for various metabolic reactions. The number of enzymes involved in the synthesis of the A4 polysaccharide from UDPG is at least seven, and for A1 polysaccharide from GDPMan and UDPG, at least eight. There is therefore considerable expenditure of energy in the synthesis of the nucleotide sugar precursors and the specific transferases required for formation of the polysaccharide. In the laboratory, the non-mucoid cell survives as well as the mucoid. If it is assumed that the cell does not always have sufficient energy and nutrients in the natural environment, then unless the capsule plays a significant role in survival, one would expect non-mucoid strains of K. aerogenes to be selected. They would not waste energy or nutrients in synthesis of a polymer unable to be re-utilized by the cell. Although one definite role was not established, the capsule of strain A1 aided in survival against dessication, against attack by organic cations and enzymes degrading the cell wall, and against phage when compared to an A1(0) strain. All these functions and any others which help to maintain the integrity of the cell and protect it in the environment may explain why all naturally occurring species of K. aerogenes are capsulate despite the fact that a capsule is not essential for viability.

No bacteria or fungi were found which possessed enzymes degrading the polysaccharide of strains A1 and A4, although a wide variety of sources were used. Indeed it has been a general finding that bacteria able to hydrolyse bacterial polysaccharides are very rare. A large number of species, such as of Myxobacteria, would be expected to utilize these polysaccharides, thereby gaining an advantage

over other organisms in that place. Perhaps other groups of micro-organisms such as protozoa or algae may be involved.

CELL-FREE SYNTHESIS OF A4 EXOPOLYSACCHARIDE

A cell-free system was developed to obtain and detect synthesis of the exopolysaccharide of strain A4. A crude cell envelope fraction was used which contains in addition to the cell wall-membrane, capsular polysaccharide material and enzymes, either particulate, attached to the cell membrane, or soluble. It may be better to utilize a mutant of A4 deficient in some known step leading to the synthesis of the nucleotide sugar precursors such as UDPGal-4-epimerase or UDPG dehydrogenase, and which would therefore contain no preformed polysaccharide in the preparation. Interconversion between nucleotide sugars would be reduced also. Incorporation of radioactivity from labelled sugar nucleotides into those fractions obtained from partial acid hydrolysis of the A4 polysaccharide was taken to represent polysaccharide synthesis. This was the most specific method which could be developed in the absence of a phage or bacterial depolymerase or of a specific antiserum to the polysaccharide. Interconversion of the sugar nucleotides such as UDPG being epimerized to UDPGal occurred during incubation indicating that the enzymes were present and active in the preparation to permit this, and that all three nucleotides may therefore not have to be present before polysaccharide may be synthesized.

Good uptake of glucose from UDPG into polysaccharide material occurred with resultant labelling of the two slowest moving fractions of the hydrolysate. Uptake of galactose from UDPGal and glucuronic acid from UDPGlUA into polysaccharide material was poor. It was not clear why glucose should be incorporated so well without concommitant incorporation of the other two sugars unless an

exchange reaction with glucose in the preformed polysaccharide was occurring or glucose was being incorporated into another polymer. The first is unlikely as glucose is in the main chain structure of the polysaccharide and not in the side chains. The second is also unlikely as the fractions of the partially hydrolysed polysaccharide labelled with glucose are probably specific to the polysaccharide, and other oligosaccharides would not be expected to have the same mobility on electrophoresis. Although conditions were suitable for incorporation of glucose into the polysaccharide, perhaps they were not for incorporation of glucuronic acid and galactose.

However pre-incubation of the reaction mixture with UDPG led to good uptake of galactose from UDPGal, and also glucuronic acid from UDPGlUA to a smaller extent. Pre-incubation may be necessary to adapt the system to accepting the nucleotide sugars and to allow time for the polymerization. Incorporation from both UDPGal and UDPGlUA was into all three hydrolysate fractions. Despite varying the times of the pre-incubation period and the temperature of incubation, total incorporation of glucuronic acid from UDPGlUA was still only about 1/10th that from UDPG. It appears that glucose is taken up at the fastest rate, followed by galactose, and little incorporation of glucuronic acid occurs so that only a small amount of complete new polymer may be synthesized. Cell envelope fractions prepared from non-mucoid mutants of A4 showed no incorporation of glucose from UDPG into polymeric material. The non-mucoid mutants are assumed to differ from A4 only in loss of the ability to synthesize exopolysaccharides.

The question arises whether an intermediate which is lipid-linked is involved in exopolysaccharide production. The repeating tetrasaccharide unit of the polymer may then be assembled on this intermediate before polymerization. A system like this has been shown in synthesis of the O-side chains of the lipopolysaccharide, first in cell-free systems and then in whole cells (Dankert,

Wright, Kelley and Robbins, 1966). It has not been demonstrated until recently in any reaction mixture synthesizing exopolysaccharide except that, during synthesis of cellulose, a glucose-lipid complex was shown to be a possible precursor of cellodextrins (Khan and Colvin, 1961). It is an attractive hypothesis because it would enable the repeating unit to be assembled within the cell membrane, transported through as the lipid-linked complex and polymerized. In addition the specificity of the polysaccharide would be retained by the enzymes assembling the repeating unit on the lipid carrier. It would also explain the "all-or-none" phenomenon of the production of the polysaccharide and why mutants were not found with altered polysaccharide structure. If one enzyme was deficient, the lipid intermediate could not be completely synthesized and no polysaccharide formed. Troy and Heath (1968) demonstrated the incorporation of galactose from UDPGal into lipid material in a cell-free system of a K. aerogenes strain. They also showed the addition of mannose from GDPMan to this lipid intermediate so that galactose and mannose were present in the ratio 1: 1. Addition of the third nucleotide sugar UDPGlUA to the reaction mixture led to incorporation of all three sugars into polysaccharide material.

Using the cell-free system of strain A4, glucose from UDPG was taken up into the chloroform/methanol fraction in the presence of UDPGal and UDPGlUA or without either. Galactose from UDPGal was incorporated to half this extent, and only slight incorporation of glucuronic acid from UDPGlUA occurred either alone or in the presence of the other two nucleotide sugars. Alteration of the incubation temperature from 15° to 25° did not change the result nor a longer period of incubation - up to 4 hours. It was concluded that either glucose and galactose were being incorporated into a lipid material for synthesis of another compound

and that no lipid-linked intermediate of the exopolysaccharide existed, or the turnover of the intermediate when glucuronic acid was labelled was so rapid that at any one time little incorporation could be detected, or incorporation from UDPGlUA occurred only slightly under the conditions of the experiment. The last seems the most probable.

The presence of ADPG in the reaction mixture slowed the rate of incorporation from UDPG into the lipid fraction. ADPG is the specific donor for glycogen synthesis. However, although glycogen formation may occur in the reaction mixture, this would not be expected to affect synthesis of the exopolysaccharide, if it is assumed that ADPG cannot be used for exopolysaccharide synthesis and UDPG for glycogen synthesis. In glycogen synthesis, no lipid-linked intermediates have been demonstrated (Gahan and Conrad, 1968). Thus why there should be such a marked effect by ADPG on the rate of uptake of glucose from UDPG is not clear, unless UDPG is utilized to a small extent for glycogen synthesis. UDPG has been shown in some bacteria to act as a glucosyl donor for glycogen synthetase with low efficiency (Greenberg and Preiss, 1964).

It seems possible that lipid-linked intermediates are involved in synthesis of exopolysaccharide in A4 as there is good correlation between the uptake of glucose and galactose into the lipid fraction and their incorporation into polysaccharide. Similarily little uptake of glucuronic acid into lipid material is paralleled by its poor incorporation into polysaccharide. However before this question is settled it would be necessary to obtain the incorporation of each sugar in turn into lipid material and to isolate the components formed. One should be able to synthesize, say a glucose-lipid intermediate, a galactose-glucose intermediate and a glucuronic-galactose-glucose intermediate depending on the sugar nucleotides added to the system. The interconversion of nuclectide

sugars would have to be prevented by use of a mutant deficient in some enzyme involved in nucleotide sugar biosynthesis. The advantage of using a cell-free system in this case is that there is no dilution of radioactivity. If whole cells were utilized considerable dilution in the soluble pool would occur and it may be almost impossible to isolate any lipid intermediates involved.

Chloramphenicol had a significant effect in lowering the incorporation of glucose into lipid material and almost stopping its incorporation into polysaccharide material. This may be further evidence that synthesis of these two compounds are linked. Chloramphenicol prevents protein synthesis and it is not clear why it should have such a large effect on polysaccharide formation. All the enzymes should be present in the reaction mixture to allow synthesis of the intermediates and the completed polysaccharide, and one wonders whether inhibition by the high concentration of chloramphenicol of any of these reactions occurs. If any regulators, repressors or inducers are involved in polysaccharide synthesis, chloramphenicol may prevent their synthesis or action.

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ABBREVIATIONS

glc	glucose
gal	galactose
man	mannose
rha	rhamnose
col	colitose
fuc	fucose
abe	abequose
tal	talose
fru	fructose
xyl	xylose
hep	heptose
KDO	2-keto-3-deoxyoctonate
NAcglc	N-acetylglucosamine
NAcgal	N-acetylgalactosamine
NAcfuc	N-acetylfucosamine
NAcmur	N-acetylmuramic acid
NAcneuraminic	N-acetylneuraminic acid
$glcNH_2$	glucosamine
$galNH_2$	galactosamine
$manNH_2$	mannosamine
$fucNH_2$	fucosamine
$hexNH_2$	hexosamine
glUA	glucuronic acid
galUA	galacturonic acid
manUA	mannuronic acid
glc-1-Ⓟ	glucose-1-phosphate

glc-6-Ⓟ	glucose-6-phosphate
man-1-Ⓟ	mannose-1-phosphate
man-6-Ⓟ	mannose-6-phosphate
fru-6-Ⓟ	fructose-6-phosphate
CMP	cytidine 5′-monophosphate
UMP	uridine 5′-monophosphate
UDP	uridine 5′-diphosphate
UTP	uridine 5′-triphosphate
GDP	guanosine 5′-diphosphate
TDP	thymidine 5′-diphosphate
AMP	adenosine 5′-monophosphate
ADP	adenosine 5′-diphosphate
ATP	adenosine 5′-triphosphate
UDPG	uridine 5′-diphosphate glucose
UDPGal	uridine 5′-diphosphate galactose
UDPRha	uridine 5′-diphosphate rhamnose
UDPGlUA	uridine 5′-diphosphate glucuronic acid
UDPGalUA	uridine 5′-diphosphate galacturonic acid
UDPIUA	uridine 5′-diphosphate iduronic acid
UDPNAcglc	uridine 5′-diphosphate N-acetylglucosamine
ADPG	adenosine 5′-diphosphate glucose
GDPM or GDPMan	guanosine 5′-diphosphate mannose
GDPCol	guanosine 5′-piphosphate colitose
GDPfuc	guanosine 5′-diphosphate fucose
GDPManUA	guanosine 5′-diphosphate mannuronic acid
TDPRha	thymidine 5′-diphosphate rhamnose
CMPKDO	cytidine 5′-monophosphate 2-keto-3-deoxyoctonate

ala	alanine
glu	glutamic acid
lys	lysine
DAP	diaminopimelic acid
gly	glycine
his	histidine
NAD	nicotinamide-adenine dinucleotide (oxidised)
NADH	nicotinamide-adenine dinucleotide (reduced)
NADP	nicotinamide-adenine dinucleotide phosphate (oxidised)
NADPH	nicotinamide-adenine dinucleotide phosphate (reduced)
FAD	flavine-adenine dinucleotide
O-Ac	O-acetyl
Ⓟ	phosphate
PP_i	pyrophosphate
LPS	lipopolysaccharide
PS	polysaccharide

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